sequence is necessary for the interaction with TCF-4. It has been consistently reported that Ku70 needs to retain its three-dimensional structure to interact with Ku80, DNA, and other proteins (23).

Ku70 suppresses TCF-4–mediated gene transcriptional activity. To investigate the functional involvement of Ku proteins in the TCF-4 and β-catenin transcriptional complex, we knocked down the expression of Ku70 using shRNA. The decreased expression of Ku70 was confirmed by Western blotting (Fig. 3*A*). The knockdown of Ku70 expression increased the luciferase activity of TOP-FLASH, the canonical reporter of TCF/LEF transcriptional activity, ~2-fold over mock transfection (Fig. 3*B, black columns*) but did not affect significantly that of the mutant reporter FOP-FLASH (Fig. 3*B, gray columns*). Conversely, overexpression of Ku70 by cDNA transfection suppressed the TOP-FLASH activity ~4-fold (Fig. 3*B, *, Ku70*). Unlike Ku70, however, knockdown of Ku80 expression did not significantly affect the TOP-FLASH or FOP-FLASH activity (data not shown). Similar enhancement of TCF/LEF transcriptional activity by knockdown of Ku70 was observed in HepG2 and Li7 cells (Supplementary Fig. S2).

Consistent with the reporter assay, knockdown of Ku70 expression by transfection of shRNA into HCT116 cells increased the expression of known downstream target genes of TCF-4, including *c-myc* (*MYC*), *cyclin D1* (*CCND1*), *ETS2*, and *MDR1* (*ABCB1*; Fig. 3*C*). The expression of Ku70 mRNA in cancer tissues (*T*) was clearly decreased in four of five cases of sporadic colorectal cancer in comparison with the corresponding normal tissues (*N*, Fig. 3*D*).

Competitive regulation of the TCF-4 and β-catenin complex by Ku70 and PARP-1. Because PARP-1 has been reported to

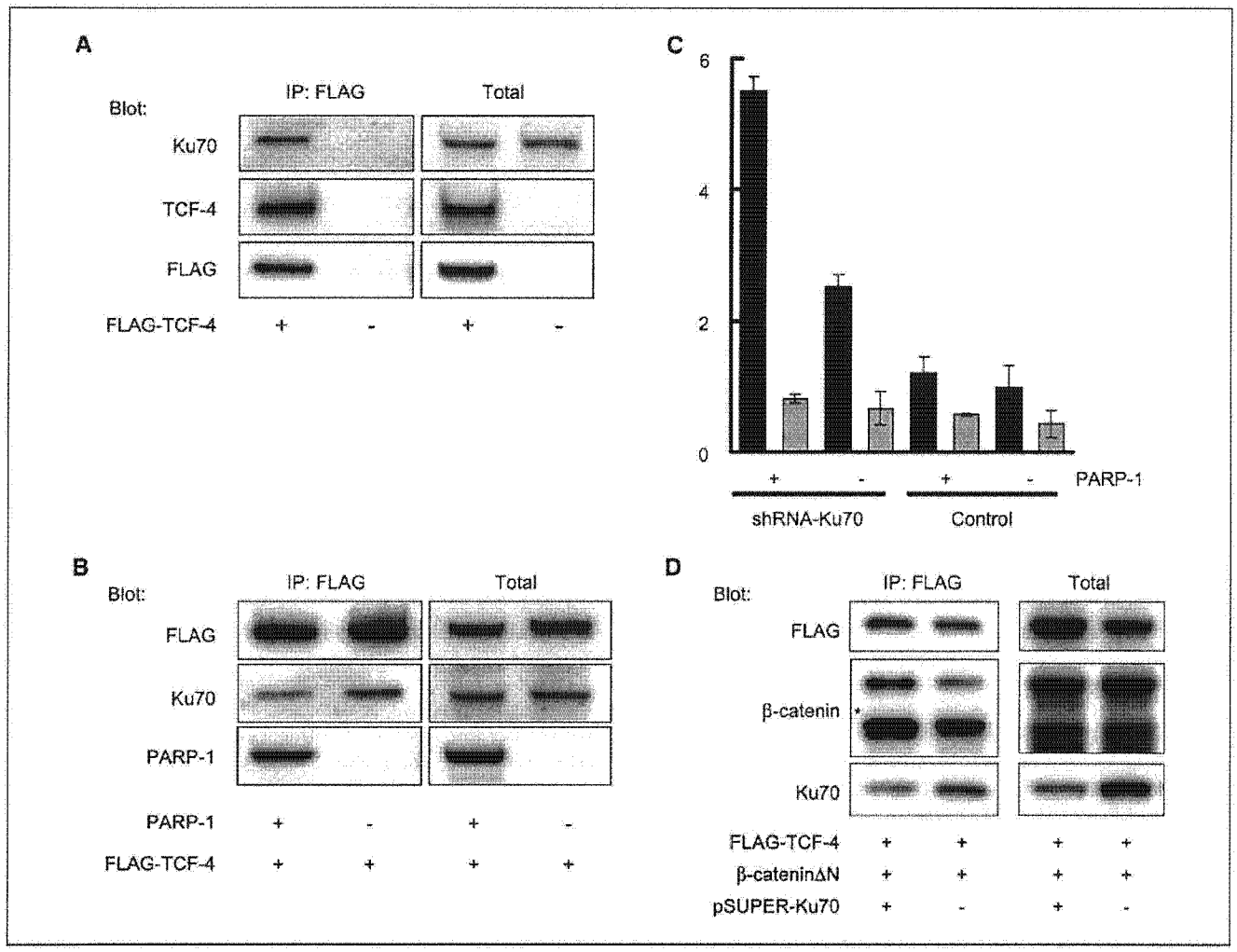

Figure 4. Competitive regulation of the TCF-4 and β-catenin complex by Ku70 and PARP-1. *A*, PARP-1–null MEF were transfected with FLAG–TCF-4 or FLAG-MOCK, and whole lysates (*Total*) and immunoprecipitates with anti-FLAG affinity beads (*IP: FLAG*) were blotted with anti-Ku70, anti-TCF-4, and anti-FLAG antibodies. *B*, PARP-1–null MEF were transfected with FLAG–TCF-4 and pcDNA3.1–PARP-1 or control pcDNA3.1. Whole lysates and immunoprecipitates with anti-FLAG affinity beads were blotted with anti-FLAG, anti-Ku70, and anti–PARP-1 antibodies. *C*, HCT116 cells were cotransfected with a mixture of pSUPER-Ku70(A) and pSUPER-Ku70(B) or empty pSUPER (*Control*) as well as pcDNA3.1-PARP-1 [*PARP-1*(+)] or empty pcDNA3.1/myc-His [*PARP-1*(−)] along with TOP-FLASH or FOP-FLASH luciferase reporter. Forty-eight hours after transfection, the luciferase activity of TOP-FLASH (*black columns*) and FOP-FLASH (*gray columns*) was measured. Activity was adjusted to the TOP-FLASH activity of the control transfectant [*Control, PARP-1*(−)] and expressed as a fold increase. *D*, HEK293 cells were transfected with FLAG–TCF-4, β-cateninΔN134, and a mixture of pSUPER-Ku70(A) and pSUPER-Ku70(B) (+) or empty pSUPER (−). Total cell lysates were immunoprecipitated with anti-FLAG affinity beads and blotted with anti-FLAG, anti–β-catenin, and anti-Ku70 antibodies.

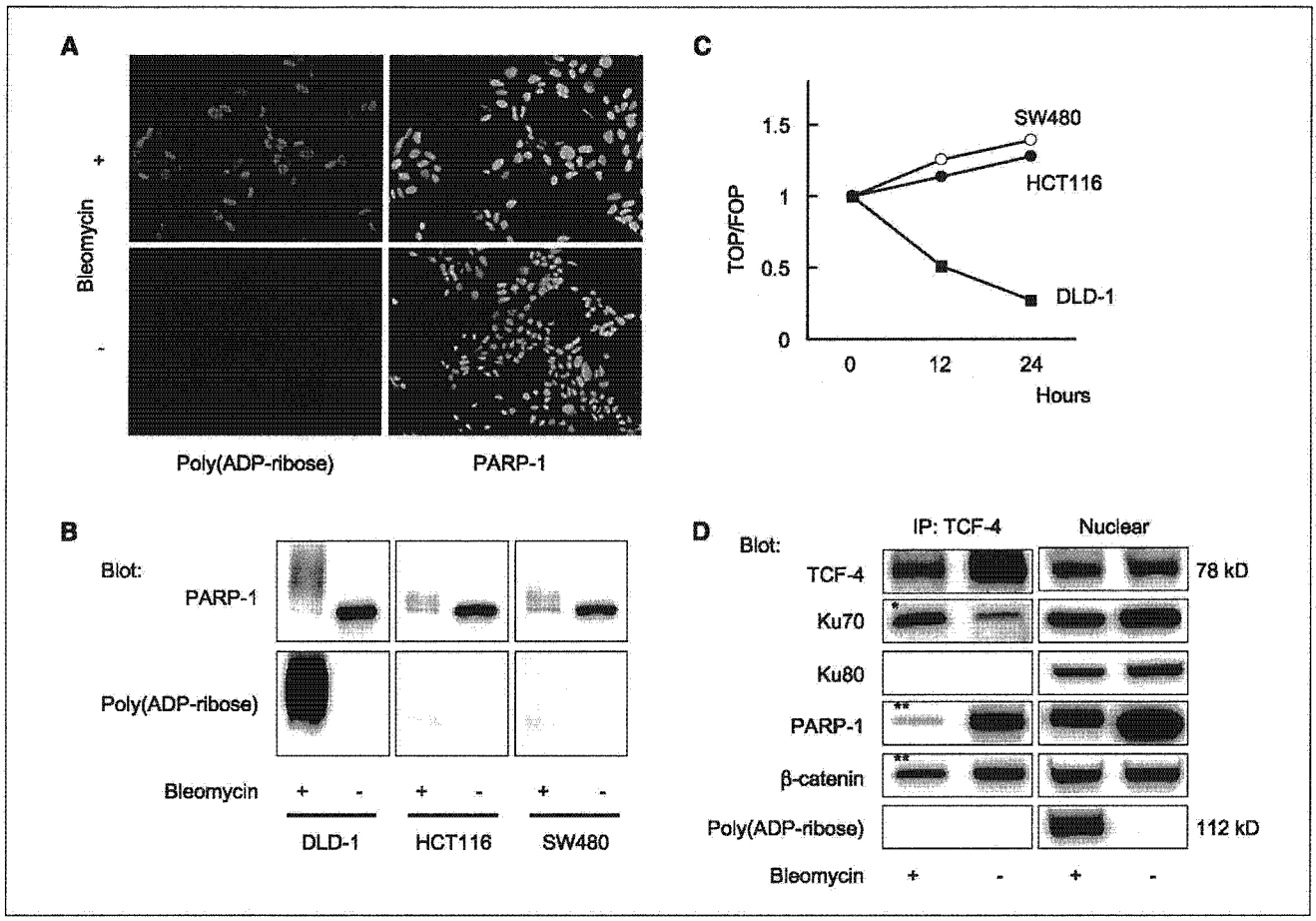

Figure 5. Possible linkage of DNA damage recognition and Wnt signaling. *A*, detection of polyADP-ribosylation in DLD-1 cells incubated with medium containing, or lacking, bleomycin for 6 h. Immunofluorescence staining was done using anti–poly(ADP-ribose) rabbit polyclonal antibody (*red*) and anti–PARP-1 mouse monoclonal antibody (*green*). *B*, detection of PARP-1 (*top*) and its polyADP-ribosylation (*bottom*) in DLD-1, HCT116, and SW480 cells untreated or treated with bleomycin for 6 h. Note that polyADP-ribosylated PARP-1 protein migrated more slowly and was less reactive with anti–PARP-1 antibody (*top*). *C*, TCF/LEF transcriptional activity of DLD-1, HCT116, and SW480 cells untreated (*0 h*) or treated only with bleomycin for 12 or 24 h. The ratio of TOP-FLASH to FOP-FLASH (*TOP/FOP*) was adjusted to that of the control (untreated) and expressed as a fold increase. *D*, DLD-1 cells were untreated (–) or treated with bleomycin for 3 h (+). Nuclear extracts (*Nuclear*) and immunoprecipitates with anti–TCF-4 antibody (*IP: TCF-4*) were blotted with anti–TCF-4, anti-Ku70, anti-Ku80, anti–PARP-1, anti–β-catenin, and anti–poly(ADP-ribose) antibodies.

interact with the Ku heterodimer (24), we used PARP-1–null MEF to investigate whether the interaction between TCF-4 and Ku proteins is mediated by PARP-1. Ku70 was coimmunoprecipitated with FLAG–TCF-4 even in the absence of PARP-1 (Fig. 4*A*), revealing that PARP-1 is not necessary for the interaction between TCF-4 and Ku. Restoration of PARP-1 did not affect the total amount of Ku70 in the nucleus (Fig. 4*B, Total*), but the amount of Ku70 coimmunoprecipitated with FLAG–TCF-4 was reduced (Fig. 4*B, IP: FLAG*), suggesting that PARP-1 competes with Ku70 for binding to TCF-4. Ku80 was barely coimmunoprecipitated with FLAG–TCF-4 in PARP-1–null MEF (data not shown).

We hypothesized that the transcriptional activity of TCF-4 is mutually regulated by the relative amount of Ku70, PARP-1, and β-catenin proteins binding to TCF-4. The enhancement of TOP-FLASH activity by transfection of Ku70 shRNA was further augmented by PARP-1 overexpression (Fig. 4*C*). Ku70 seems to suppress the transcriptional activity of TCF-4 by inhibiting the participation of β-catenin in the transcriptional complex containing TCF-4. Knockdown of Ku70 expression did not affect the total amount of β-catenin in the nucleus (Fig. 4*D, Total*), but the amount of β-catenin protein coimmunoprecipitated with FLAG-TCF4 was increased (Fig. 4*D, IP: FLAG*).

Possible linkage of DNA damage recognition and Wnt signaling. When DNA is damaged, PARP-1 polyADP-ribosylates several acceptor proteins. Treatment of colorectal cancer DLD-1 cells with bleomycin, a DNA-damaging alkylating agent, induced the accumulation of polyADP-ribosylated molecules in the nucleus (Fig. 5*A*). PARP-1 polyADP-ribosylates its own automodification domain in response to DNA damage. Bleomycin induced polyADP-ribosylation of PARP-1 protein most significantly in DLD-1 cells [Fig. 5*B*, poly(ADP-ribose)]. In parallel with the degree of PARP-1 polyADP-ribosylation, bleomycin inhibited the TCF/LEF activity of DLD-1 cells but not that of SW480 and HCT116 cells (Fig. 5*C*).

Because PARP-1 competes with Ku70 for binding to TCF-4 (Fig. 4*B*) and polyADP-ribosylation inhibits the interaction of PARP-1 with TCF-4 (11), we investigated how the polyADP-ribosylation of PARP-1 affects the composition of the TCF-4–containing transcriptional complex. Nuclear extracts from DLD-1 cells untreated or treated with bleomycin were immunoprecipitated

with anti–TCF-4 antibody. Although the total amount of Ku70 in the nucleus was not affected by bleomycin treatment (Fig. 5*D*, *Nuclear*), the amount of Ku70 coimmunoprecipitated with the anti–TCF-4 antibody was significantly increased (Fig. 5*D*, *). On the other hand, the amounts of PARP-1 and β-catenin coimmunoprecipitated with anti–TCF-4 antibody were decreased (Fig. 5*D*, **).

Immunohistochemical analysis revealed the frequent presence of nuclear poly(ADP-ribose) formation in the nuclei of colorectal adenoma cells (*T*) from FAP patients, whereas this was rarely observed in normal intestinal epithelial cells (Fig. 6, *N*).

Discussion

In this study, we showed that Ku70 and Ku80 are native components of the TCF-4 and β-catenin transcriptional complex (Fig. 1*D*). Ku70 physically interacts with a domain of TCF-4 containing the HMG box (Fig. 2*A*). Ku70 was an inhibitor of the TCF/LEF transcriptional activity (Fig. 3*B*; Supplementary Fig. S2). Down-regulation of Ku70 by RNA interference increased the expression of several known target genes of TCF/LEF (Fig. 3*C*), and the expression of Ku70 mRNA was frequently down-regulated in colorectal cancer tissues (Fig. 3*D*). Consistent with our findings, down-regulation of Ku70 protein expression has been reported previously in colorectal adenoma and carcinoma (25).

Ku has already been shown to work as a transcription factor that binds to promoter elements in a sequence-specific manner (26). Ku is capable of associating with the RNA polymerase II complex (27), but the entire Ku70/Ku80/DNA-PKcs complex is thought to be required for transcriptional regulation. DNA-PK phosphorylates RNA polymerase I (28) and II (29). Furthermore, DNA-PK interacts and/or phosphorylates other oncogenic transcription factors, including c-myc (30) and c-jun (31). However, these DNA-PK–induced phosphorylation mechanisms seem inadequate to explain the regulation of TCF-4 and β-catenin–mediated gene transactivation by Ku70, because the native TCF-4 and β-catenin complex contained mainly Ku70 (Fig. 1*D*). Ku80 is necessary for the recruitment and activation of DNA-PKcs (32), but knockdown of Ku80 by RNA interference did not affect the transcriptional activity of TCF-4 (data not shown). Ku70 is expressed in the nucleus, whereas Ku80 and DNA-PKcs are expressed either exclusively or predominantly in the cytoplasm of colorectal adenoma and carcinoma cells (25). A previous study has shown that the Ku heterodimer interacts with YY1 and suppresses α-myosin heavy-chain gene expression independently of DNA-PKcs (33).

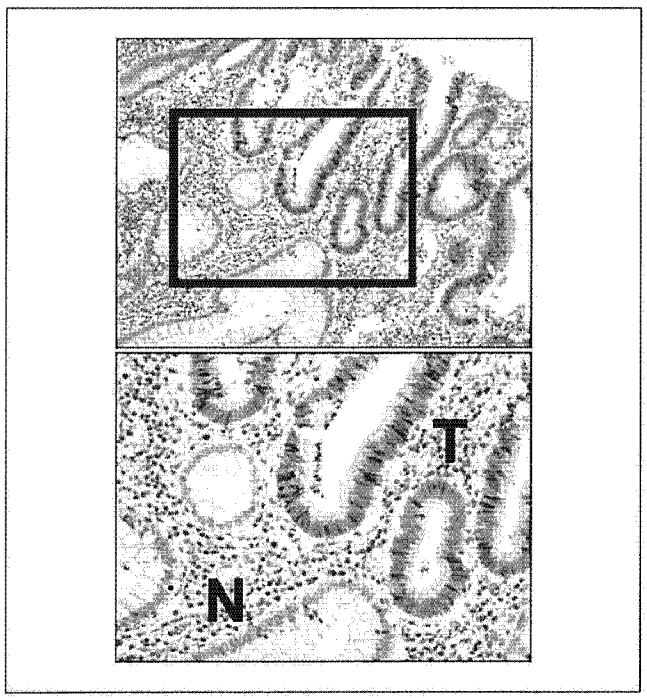

Figure 6. Nuclear poly(ADP-ribose) formation in colorectal adenoma. Immunohistochemistry of colorectal adenoma and normal glands of a FAP patient. Formation of poly(ADP-ribose) was detected with anti–poly(ADP-ribose) polyclonal antibody.

We previously reported that PARP-1 is a native component of the TCF-4 and β-catenin complex and that PARP-1 physically interacts with the region of TCF-4 distal to the HMG box (11). PARP-1 has already been reported to form a complex with the Ku heterodimer (34). However, the interaction of Ku70 with TCF-4 is not mediated by PARP-1, and, in fact, PARP-1 competes with Ku70 for binding to TCF-4. We observed that Ku70 was coimmunoprecipitated with TCF-4 even in PARP-1–null cells (Fig. 4*A*). Transfection of PARP-1 decreased the amount of Ku70 present in the immunoprecipitate with anti–TCF-4 antibody (Fig. 4*B*). The domain of TCF-4 binding to Ku70 (Fig. 2*A*) was physically close to the domain binding to PARP-1 (11). In contrast to Ku70, PARP-1 was overexpressed in colorectal cancer (11) and enhanced the transcriptional activity of TCF/LEF. Although transfection of Ku70 shRNA or cDNA alone had a small effect (~2- to 4-fold) on the TCF/LEF transcriptional activity (Fig. 3*B*; Supplementary Fig. S2), the combination of PARP-1 overexpression and Ku70 down-regulation markedly increased its activity (by >5-fold; Fig. 4*C*). The transcriptional activity of TCF-4 seems to be competitively regulated by the relative amount of Ku70 and PARP-1 proteins binding to TCF-4 (Fig. 5*D*).

PARP-1 is activated by DNA strand breakage and facilitates DNA repair by polyADP-ribosylating various acceptor molecules as well as its own automodification domain. Without DNA damage, the amount of polyADP-ribosylated proteins is kept at a low level (Fig. 5*A*). Poly(ADP-ribose) formation was barely observed in normal colon epithelial cells, whereas colorectal adenoma cells frequently accumulated nuclear poly(ADP-ribose) (Fig. 6). DNA damage is caused by endogenous free radicals produced as byproducts of oxidative metabolism. We previously reported that a key redox-status regulatory protein, manganese superoxide dismutase, was overexpressed even in small adenomas of FAP patients in parallel with the accumulation of β-catenin (22), indicating the occurrence of a certain type of DNA damage during the course of early colorectal carcinogenesis.

The protein composition of the TCF-4–containing nuclear complex is not fixed but regulated dynamically in response to DNA damage. Based on the present observations and previous studies, we propose a working hypothesis that the transcriptional activity of TCF-4 is regulated by polyADP-ribosylation of PARP-1 and subsequent recruitment of Ku70 to TCF-4 (Supplementary Fig. S3). In response to DNA damage, PARP-1 polyADP-ribosylates its own automodification domain (Fig. 5*B*). This modification inhibits the interaction between PARP-1 and TCF-4 (11), and the dissociation of PARP-1 from TCF-4 allows Ku70 to interact with TCF-4. The amount of β-catenin coimmunoprecipitated with TCF-4 was regulated by Ku70 (Fig. 4*D*). The recruitment of Ku70 into TCF-4 likely inhibits the interaction between TCF-4 and

β-catenin, the transcriptional activity of TCF-4, and the expression of target genes of TCF-4.

In summary, we have revealed that Ku70 and PARP-1 regulate TCF-4 and β-catenin–mediated gene transactivation in a competitive manner. Although our model may be oversimplified, identification of cross-talk between the Wnt signaling pathway and DNA damage recognition will provide a novel insight into the mechanism of colorectal carcinogenesis and suggest possible avenues of therapeutic intervention.

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Involvement of Splicing Factor-1 in β-Catenin/T-Cell Factor-4-Mediated Gene Transactivation and Pre-mRNA Splicing

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Background & Aims: **β-Catenin is the downstream effector of the Wnt signaling pathway and is involved in the process of colorectal carcinogenesis. However, it is still uncertain whether β-catenin exerts its oncogenic function solely by coactivating the target genes of T-cell factor-4 (TCF4). We previously reported that the β-catenin/TCF4 complex contains several classes of RNA-binding proteins and regulates the premessenger RNA splicing reaction, but the identity of the exact effector molecule downstream of the β-catenin/TCF4 complex has not been established.** ***Methods:*** **Using isotope-coded affinity tagging and mass spectrometry, we examined more than 4000 peptides derived from colorectal cancer cells and identified that splicing factor-1 (SF1) was one of the proteins whose expression is regulated by the β-catenin/TCF4 complex.** ***Results:*** **The expression of SF1 was found to be correlated with the differentiation status of intestinal epithelial cells and inversely correlated with tumorigenesis. Immunoprecipitation and immunofluorescence microscopy revealed that SF1 was a complex, and β-catenin-evoked gene transactivation and cell proliferation were negatively regulated by SF1 complementary DNA transfection. SF1 was essential for the induction of alternative splicing by the β-catenin/TCF4 complex, and SF1 complementary DNA transfection induced known cancer-related splice variants, such as Wnt-induced secreted protein-1v and fibroblast growth factor receptor-3-ATII.** ***Conclusions:*** **The β-catenin/TCF4 complex regulates the level of SF1 protein expression, and, conversely, SF1 interacts with the complex and regulates its gene transactivation and premessenger RNA splicing activities. Identification of the interaction may shed light on a novel aspect of the Wnt signaling pathway.**

The Wnt signaling pathway regulates cell fate, differentiation, proliferation, and death, and thus plays critical roles in embryonic development and carcinogenesis.[1–4] The Wnt signal is transmitted from the membrane receptors to the cytoplasmic multiprotein complex containing the adenomatous polyposis coli (*APC*) gene product, axin/axil, glycogen synthase kinase-3β (GSK-3β), and β-catenin.[5,6] This complex acts as a molecular chaperone that mediates the phosphorylation of β-catenin by GSK-3β, and the phosphorylated β-catenin protein is ubiquitinated and rapidly degraded.[7,8] More than 80% of colorectal cancers carry mutations in the *APC* gene,[9,10] and half of the remainder contain mutations in the β-catenin (*CTNNB1*) gene.[11,12] These mutations cause dysfunction of the chaperone, which results in the accumulation of cytoplasmic β-catenin protein.[13] The accumulated β-catenin is thought to cause colorectal carcinogenesis by forming a complex with T-cell factor-4 (TCF4), a member of the TCF/lymphoid enhancer factor (LEF) family, and by coactivating the target genes of TCF4.[14]

Several immediate transcriptional targets of TCF4 have been identified using complementary DNA (cDNA)/oligonucleotide microarrays,[15–19] but the entire protein network of β-catenin-mediated intestinal carcinogenesis seems complicated and has not yet been fully explained. In the present study, we used a liquid chromatography and mass spectrometry (LC-MS)-based quantitative proteomic approach[20] to identify comprehensively a population of proteins whose expression is regulated by the β-catenin/TCF4 complex in colorectal cancer cells. As a result, we identified splicing factor-1 (SF1) as one of the proteins whose expression is negatively regulated by the β-catenin/TCF4 complex, and its expression was found to be directly correlated with the differentiation status of intestinal epithelial cells and inversely correlated with tumorigenesis. SF1 functioned as a component of the β-catenin/TCF4 complex and negatively regulated β-catenin-evoked gene transactivation and cell proliferation.

Abbreviations used in this paper: ER, estrogen receptor; FAP, familial adenomatous polyposis; FGFR, fibroblast growth factor receptor; FUS, fusion; GAPDH, glyceraldehyde-3 phosphate dehydrogenase; GSK-3β, glycogen synthase kinase-3β; HEK, human embryonic kidney; hnRNP, heterogeneous nuclear ribonucleoprotein; ICAT, isotope-coded affinity tagging; LC, liquid chromatography; LEF, lymphoid enhancer factor; MS, mass spectrometry; MS/MS, tandem mass spectrometry; SF1, splicing factor-1; TCF, T-cell factor; VAMP, vesicle-associated membrane protein; WISP, Wnt-induced secreted protein.

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We previously reported that the β-catenin/TCF4 complex contains several classes of RNA-binding proteins, including fusion (FUS) and heterogeneous nuclear ribonucleoproteins (hnRNPs), and regulates the pre-messenger RNA (mRNA) splicing reaction.[21] However, the exact effector molecule downstream of the β-catenin/TCF4 complex had not been identified. Here, we report finding that SF1 is essential for the induction of alternative splicing by the β-catenin/TCF4 complex. Human cancers express a large number of alternatively spliced transcripts.[22] SF1 may play an important role in the process of carcinogenesis.

Materials and Methods

Cell Lines

Human embryonic kidney (HEK) cell line 293 and human colorectal cancer cell line DLD-1 were obtained from the Health Science Research Resources Bank (Osaka, Japan). Human cervical cancer cell line HeLa and simian kidney epithelial cell line Cos-7 were purchased from the Riken Cell Bank (Tsukuba, Japan). Human colorectal cancer cell line HCT-116 was purchased from the American Type Culture Collection (Rockville, MD).

Plasmid Construction and Establishment of DLD1 Tet-off TCF4BΔN30

AU1-tagged TCF4B cDNA (nucleotides 398–2138, accession number Y11306) was subcloned into pTRE2-pur (BD Biosciences, San Jose, CA) to create pTRE2-TCF4BΔN30. DLD1 cells were double transfected sequentially with regulatory pTet-OFF (BD Biosciences) and responsive pTRE2-TCF4BΔN30 or pTRE2-pur control plasmid.

The human SF1 expression construct (pcDNA-3.1-His-SF1) was kindly provided by Dr. M. A. Garcia-Blanco (Duke University Medical Center).[23] Full-length human β-catenin cDNA and its truncated form (lacking a 134-amino acid sequence in its NH_2-terminus[15]) were subcloned into pFLAG-CMV4 (Sigma-Aldrich, St Louis, MO) to prepare pFLAG-β-catenin and pFLAG-β-cateninΔN134, respectively. Human TCF4E cDNA lacking a 30-amino acid β-catenin-binding site in its NH_2-terminus[14] was subcloned into pFLAG-CMV4 to prepare pFLAG-TCF4EΔN30. The composition of all constructs described in this study was confirmed by restriction endonuclease digestion and sequencing.

Isotope-Coded Affinity Tagging Analysis

DLD1 Tet-off TCF4BΔN30 cells were cultured for 7 days in the absence or presence of 0.1 μg/mL doxycycline (Dox). The cells were washed with ice-cold PBS, allowed to swell for 15 minutes in hypotonic buffer (50 mmol/L Tris-HCl, pH 7.4, 5 mmol/L $MgCl_2$, 5 mmol/L $CaCl_2$) supplemented with protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN), and then centrifuged for 60 minutes at 105,000*g* to separate the soluble fraction (fraction 1). The pellet was resuspended in lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L $CaCl_2$, and protease inhibitor cocktail) and homogenized. The cell homogenates were fractionated into fractions 2–5 by sequential differential centrifugation (see Supplementary Figure S1 online at www.gastrojournal.org), and each fraction was dissolved in dissolving buffer (50 mmol/L Tris-HCl, pH 8.3, 5 mmol/L EDTA, 0.5% SDS, 1% octyl glucoside, 8 mol/L urea). After adjusting the concentration of urea to 5 mol/L, 300-μg protein samples were reduced with 2 mmol/L tris (2-carboxyethyl)phosphine (TCEP) for 30 minutes at 37°C and differentially labeled with the isotopically light ($^{12}C_0$) or heavy ($^{13}C_9$) acid-cleavable isotope-coded affinity tagging (ICAT) reagent (Applied Biosystems, Foster City, CA). The $^{12}C_0$- and $^{13}C_9$-labelled samples were combined, digested with modified trypsin (Promega, Madison, WI), fractionated via cation-exchange chromatography, and purified by avidin-affinity chromatography.

The ICAT-labeled peptides were concentrated and desalted on a 500-μm ID × 1 mm HiQ sil C18-3 trapping column (KYA Technologies, Tokyo, Japan) before loading onto a 150-μm ID × 5-cm C18W-3 separation column (KYA Technologies). Peptides were then fractionated with an acetonitrile gradient (0%–80%, 200 nL/minute for 3 hours) and analyzed with a Q-Star Pulsar-*i* mass spectrometer equipped with a nanospray ionization source (Applied Biosystems). Data were processed with ProICAT software (Applied Biosystems).

Antibodies

Anti-78-kilodalton glucose-regulated protein (clone 40), antinucleoporin p62 (clone 63), and anti-β-catenin (clone 14) mouse monoclonal antibodies were purchased from BD Transduction Laboratories (Palo Alto, CA). Anti-alcohol dehydrogenase rabbit (sc-22750), antilaminin receptor rabbit (sc-20979), anti-SF1 goat (sc-21157), anti-β-catenin goat (sc-1496), anti-FUS goat (sc-8531), anti-Wnt-induced secreted protein-1 (WISP1) rabbit (sc-25441), and anti-WISP2 goat (sc-8867) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antialdolase goat polyclonal antibody (100–1141) was obtained from Rockland (Gilbertsville, PA). Antihemeoxygenase-2 rabbit polyclonal antibody (OSA-200) was purchased from Stressgen (British Columbia, Canada). Anti-Bcl-2 mouse monoclonal antibody (clone 124) was obtained from Dako (Glostrup, Denmark). Antivesicle-associated membrane protein (VAMP)-associated protein A rabbit polyclonal antibody (AB5741) was purchased from Chemicon (Temecula, CA). Anti-β-actin mouse monoclonal antibody (AC-74) and anti-SF1 goat polyclonal antibody (S1945) were obtained from Sigma-Aldrich. Anti-TCF4 (6H5-3) and anti-TCF-3/4 (6F12-3) monoclonal antibodies were purchased from Upstate (Charlottesville, VA). Anti-poly (ADP-ribose) polymerase

(PARP) rabbit polyclonal antibody (No. 9542) was purchased from Cell Signaling (Boston, MA). Anti-SF1 (AB/SF20) rabbit polyclonal antibody was purchased from CeMines (Golden, CO). Anti-FUS rabbit polyclonal antibody was generously provided by Dr. K. Shimizu (National Cancer Center Research Institute, Tokyo, Japan).

Immunoprecipitation

Nuclear extracts were prepared with the CelLytic nuclear extraction kit (Sigma-Aldrich). The extracts were incubated at 4°C overnight with anti-β-catenin monoclonal antibody, anti-SF1 goat polyclonal antibody, anti-FUS rabbit polyclonal antibody, anti-TCF4 monoclonal antibody, normal mouse IgG, normal goat IgG, or normal rabbit IgG and precipitated with Dynabeads protein G (Dynal Biotech, Oslo, Norway). The immunoprecipitated proteins were released by boiling in SDS loading buffer for 5 minutes and fractionated by SDS-PAGE.

Immunoblot Analysis

Protein samples were fractionated by SDS-PAGE and blotted onto Immobilon-P membranes (Millipore, Billerica, MA). After incubation with the primary antibodies at 4°C overnight, the blots were detected with the relevant horseradish peroxidase-conjugated anti-mouse, anti-rabbit, anti-goat, or anti-rat IgG antibody and ECL Western blotting detection reagents (Amersham Biosciences, Amersham, United Kingdom).

Tissue Samples

Male Min mice (C57BL/6J-*Apc*$^{Min/+}$) were obtained from the Jackson Laboratory (Bar Harbor, ME). Eleven

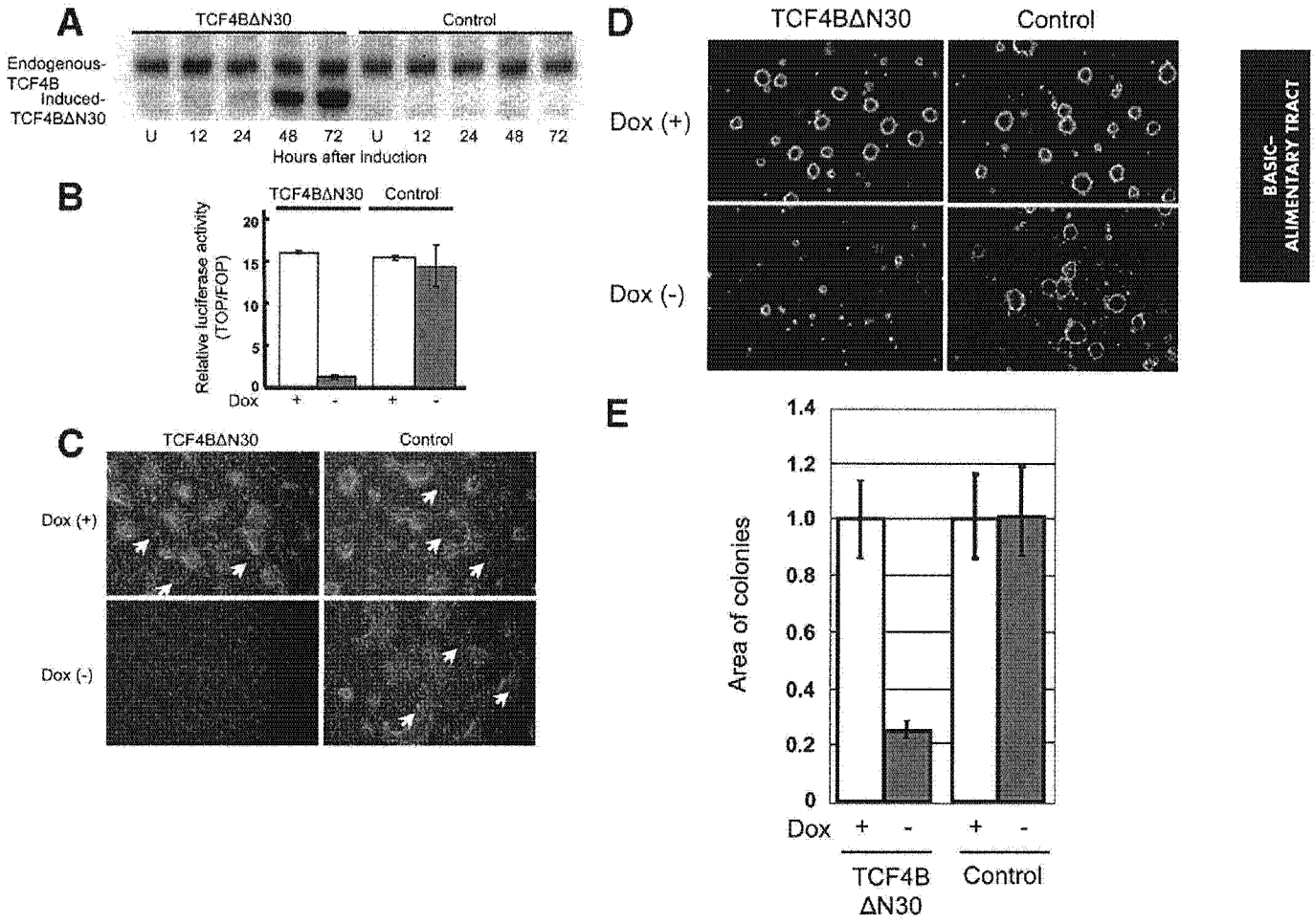


Figure 1. Establishment of DLD1 Tet-off TCF4BΔN30 and control. (*A*) DLD1 Tet-off TCF4BΔN30 (*left*) and control (*right*) cells were cultured with Dox (*U*) or without Dox for 12, 24, 48, 72 hours. Nuclear extracts were separated by SDS-PAGE and blotted with anti-TCF3/4 antibody. (*B*) DLD1 Tet-off TCF4BΔN30 (*left*) and control (*right*) cells were transfected in triplicate with canonical (TOP-FLASH) or mutant (FOP-FLASH) TCF/LEF luciferase reporters and cultured in the presence (+) or absence (−) of Dox. Luciferase activity was measured 48 hours after transfection. *Columns* represent the ratios of luciferase activity driven by TOP-FLASH to that of FOP-TOP. *Bars*, SD. (*C*) Phase-contrast microscopy of DLD1 Tet-off TCF4BΔN30 (*left*) and control (*right*) cells cultured in the presence (+) or absence (−) of Dox. *Arrows* point to the localization of piled-up foci. (*D* and *E*) Soft agar colony formation of DLD1 Tet-off TCF4BΔN30 (*left*) and control (*right*) cells cultured in the presence (+) or absence (−) of Dox (*D*). The areas occupied by colonies (*E*) are expressed as ratios to Dox (+). *Bars*, SD.

colorectal cancer cases were selected from the surgical pathology archive panel of the National Cancer Center Hospital (Tokyo, Japan). Paraffin-embedded intestinal tissue was stained by the avidin-biotin complex method. Protein samples were extracted from the small intestine and polyp tissues of a Min mouse as described previously.[15] Animal experiments were reviewed and approved by the Ethics Committee of the National Cancer Center Research Institute (Tokyo, Japan).

Immunofluorescence Cytochemistry

Cells cultured on glass coverslips (Asahi Technoglass Corp., Tokyo, Japan) were fixed with 4% paraformaldehyde at room temperature for 10 minutes and permeabilized with 0.2% Triton X-100. After blocking with 10% normal swine serum (Vector Laboratory, Inc., Burlingame, CA), the cells were incubated with anti-β-catenin mouse monoclonal and anti-SF1 goat polyclonal antibodies at 4°C overnight. After incubation with Alexa Fluor-594 anti-mouse IgG and Alexa fluor-488 anti-goat IgG antibodies (Invitrogen, Carlsbad, CA), the specimens were examined with a laser scanning microscope (LSM5 PASCAL, Carl Zeiss, Jena, Germany).[24]

Luciferase Reporter Assay

A pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate), was used to evaluate TCF/LEF transcriptional activity. Cells were transiently transfected in triplicate with one of the luciferase reporters and phRL-TK (Promega) by using the Lipofectamine 2000 reagent (Invitrogen). Luciferase activity was measured with the Dual-luciferase Reporter Assay system (Promega).

Colony Formation Assay

HEK293 cells were transfected with pcDNA3.1-His-SF1 and/or pFLAG-β-cateninΔN134 or appropriate empty plasmids by using the Lipofectamine 2000 reagent, and, 24 hours later, 750 μg/mL G418 (Geneticin; Invitrogen) was added to the culture medium. Cells were stained with Giemsa stain solution (Wako, Osaka, Japan) after 8 days of selection. A soft-agar colony formation assay was performed as described previously.[25]

In Vivo Splicing Analysis and RNA Interference

Cells were cotransfected with 0.25 μg pCS3-MT-E1A, which carries the adenovirus E1A minigene[26] (kindly provided by Dr. F. Moreau-Gachelin, Institut Curie, Paris, France, and obtained through Dr. N. Ohkura, National Cancer Center Research Institute), and pFLAG-CMV4 TCF4EΔN30, pcDNA3.1-His-SF1, or the various small interfering RNAs (siRNA) (siGENOME duplex, D-012662; Non-Specific Control Duplex X, VI) (Dharmacon, Lafayette, CO) by using Lipofectamine 2000 reagent, and, 48 hours later, the total RNA was extracted and

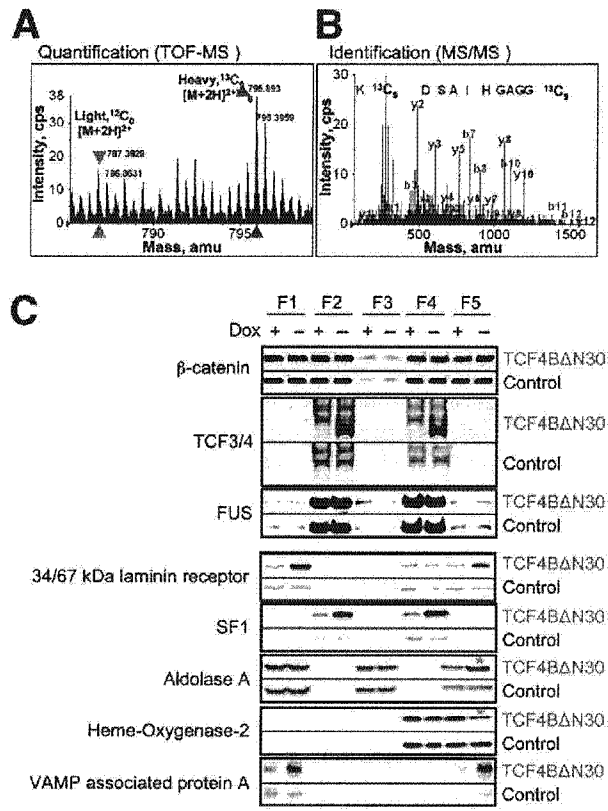


Figure 2. Identification of downstream proteins of the β-catenin/TCF4 complex. DLD1 Tet-off TCF4BΔN30 cells were cultured in the presence or absence of Dox for 7 days. The whole protein extracts were fractionated by a differential centrifugation technique as described in Supplementary Figure 1 (see Supplementary Figure 1 online at www.gastrojournal.org), labeled differentially with the $^{12}C_0$ (Dox [+]) or heavy $^{13}C_9$ (Dox [−]) ICAT reagent, and analyzed by nanoflow LC-MS (*A*) and MS/MS (*B*). cps, count(s) per second; amu, atomic mass unit(s). (*A*) MS spectrum showing the light ($^{12}C_0$)- and heavy ($^{13}C_9$)-labeled peptides of SF1 protein. The ratio of the intensity of the $^{13}C_9$ peak (indicated by *blue arrows*, 795.8936 m/z) to the intensity of the $^{12}C_0$ peak (indicated by *red arrows*, 787.3929 m/z) was 2.2042. (*B*) MS/MS spectrum of a double-charged $^{13}C_9$-labeled 795.8936 m/z-peptide matched with the CGGAGHIASDCK amino acid sequence of the SF1 protein. (*C*) Confirmation of differential expression and protein identification. Protein sample fractions (F1-5) of DLD1 Tet-off TCF4BΔN30 and control cells cultured for 7 days in the presence (+) or absence (−) of Dox were separated by SDS-PAGE and blotted with anti-β-catenin, anti-TCF3/4, anti-FUS, anti-34/67-kilodalton laminin receptor, anti-SF1, antialdolase A, antihemeoxygenase-2, and anti-VAMP-associated protein A antibodies.

analyzed by reverse transcription (RT)-polymerase chain reaction (PCR) with a pair of primers, 5′-GAGCTT-GGGCGACCTCA-3′ (RR67) and 5′-TCTAGACACAGGT-GATGTCG-3′ (E1A2). Quantification was performed with a LAS-3000 scanner and Science Lab 2003 software (Fujifilm, Tokyo, Japan).

Conventional RT-PCR Analysis

Total RNA was prepared from cell lines with the RNeasy Mini Kit (Qiagen, Valencia, CA). DNase-I-

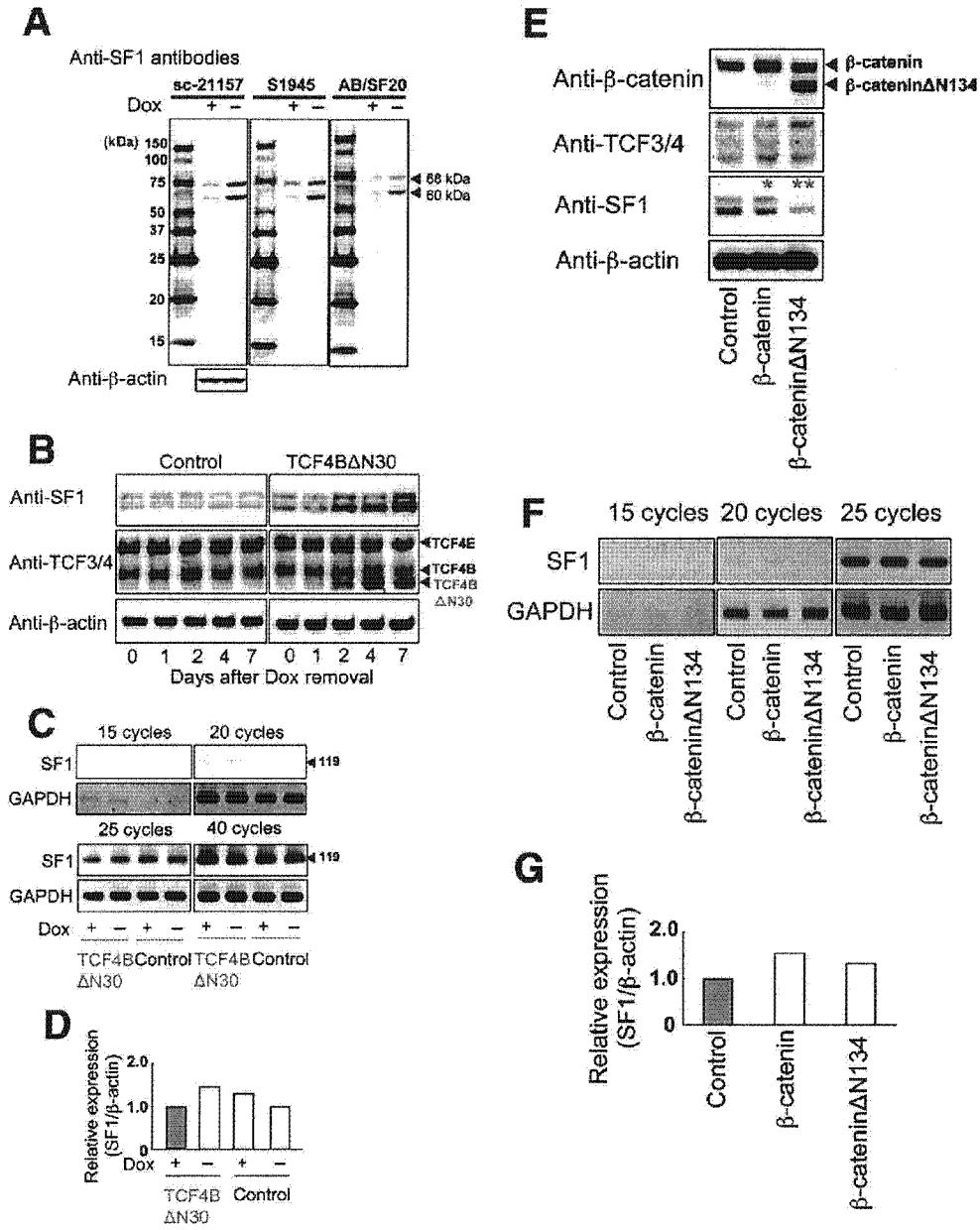


Figure 3. Posttranscriptional regulation of SF1 by the β-catenin/TCF4 complex. (*A*) Nuclear proteins extracted from DLD1 Tet-off TCF4BΔN30 cells cultured in the absence (−) or presence of Dox (+) for 7 days were separated by SDS-PAGE and blotted with 3 anti-SF1 antibodies (*left*, sc-21157; *middle*, S1945; *right*, AB/SF20) and anti-β-actin antibody (*bottom*, loading control). Anti-SF1 antibody (sc-21157) was used in all other experiments. (*B*) Nuclear proteins extracted from DLD1 Tet-off TCF4BΔN30 or control cells cultured in the absence (0) or presence of Dox for 1, 2, 4, and 7 days were separated by SDS-PAGE and blotted with anti-SF1, anti-TCF3/4, and anti-β-actin (loading control) antibodies. (*C* and *D*) Total RNA was extracted from DLD1 Tet-off TCF4BΔN30 and control cells cultured in the presence (+) or absence (−) of Dox for 7 days. Expression of SF1 and GAPDH (internal control) mRNA was detected by RT-PCR (*C*), and the expression level of SF1 relative to β-actin (ΔC_T) was measured by real-time PCR and expressed as ratios ($\Delta\Delta C_T$) to the value in DLD1 Tet-off TCF4BΔN30 cells cultured in the presence (+) of Dox (*D*). (*E*) Nuclear proteins extracted from HEK293 cells transfected with empty pFLAG-CMV4, pFLAG-β-catenin (β-catenin), or pFLAG-β-cateninΔN134 (β-cateninΔN134) were analyzed by immunoblotting with anti-β-catenin, anti-TCF3/4, anti-SF1, and anti-β-actin antibodies. (*F* and *G*) Total RNA was extracted from HEK293 cells transfected with empty pFLAG-CMV4, pFLAG-β-catenin (β-catenin), or pFLAG-β-cateninΔN134 (β-cateninΔN134). Expression of SF1 and GAPDH (internal control) mRNA was detected by RT-PCR (*F*), and the expression level of SF1 relative to β-actin (ΔC_T) was measured by real-time PCR and expressed as ratios ($\Delta\Delta C_T$) to the value in the control transfection (*G*).

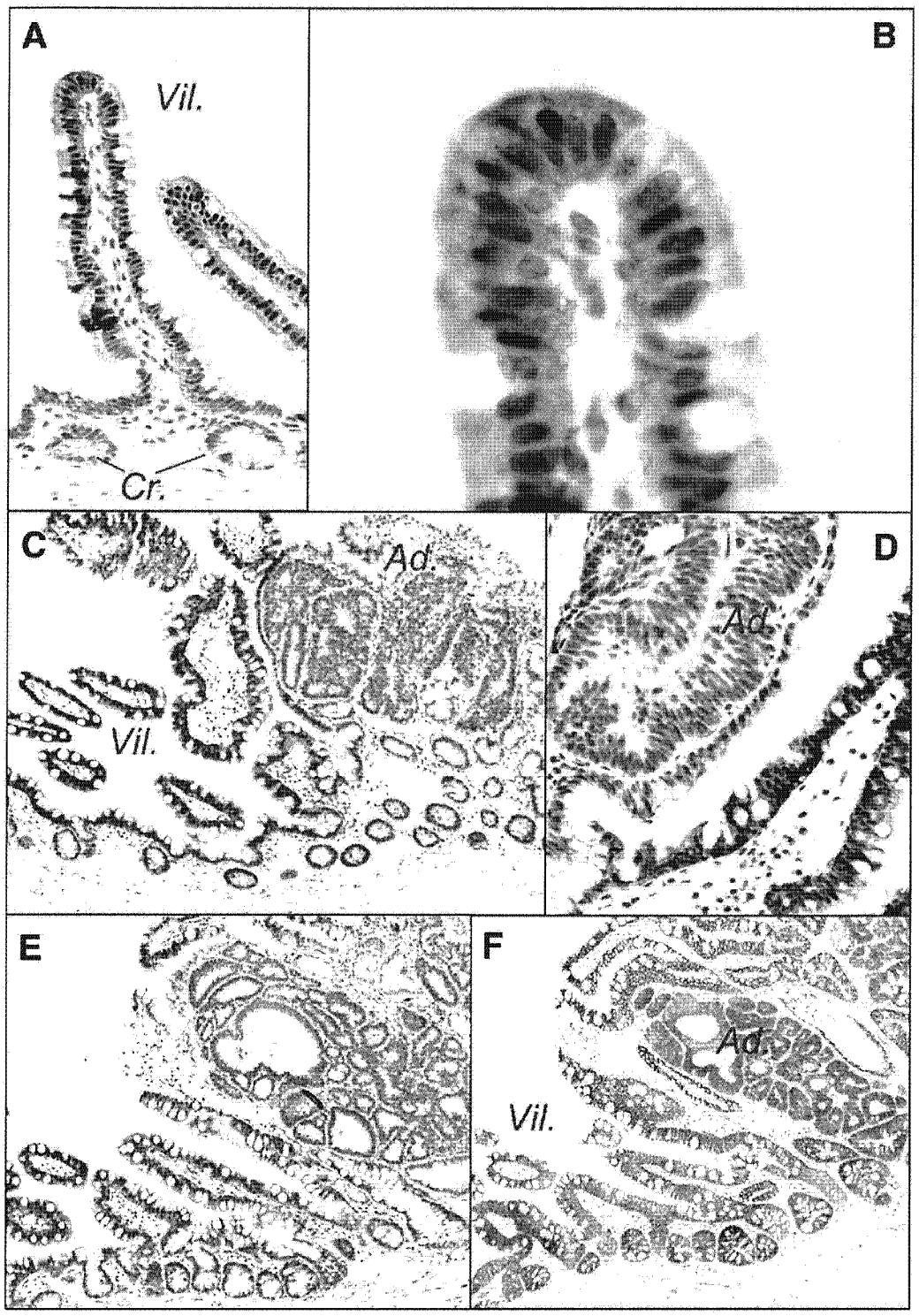


Figure 4. Expression of SF1 in intestinal tissue. (*A–F*) Immunoperoxidase staining of SF1 (*A–E*) and β-catenin (*F*) proteins in normal small intestinal (*A* and *B*) and tumor (*C–F*) tissues of Min ($Apc^{Min/+}$) mice. SF1 protein stained intensely in differentiated intestinal villous cells (*Vil.*; *A* and *B*) but was undetectable in normal undifferentiated crypts (*Cryp.*; *A*) and adenoma cells (*Ad.*; *C* and *D*). The intestinal villus and crypts in *A* are shown enlarged in *B*. (*G–L*) Immunoperoxidase staining of SF1 protein in the normal large intestine (*G*, *I*, and *J*), adenoma (*H*), and carcinoma (*K* and *L*) tissues of a Min mouse (*G* and *H*) and of colorectal cancer patients (*I–L*). *Arrows* in *G* indicate the nuclear expression of SF1 protein in normal surface epithelial cells. Tubular intestinal gland cells (crypts of Lieberkühn) (*G* and *I*) and adenoma cells (*Ad.*; *H*) lack expression of SF1. The area circumscribed by the *orange box* in (*I*) has been enlarged to demonstrate the nuclear expression of SF1 protein in normal surface epithelial cells (*J*).

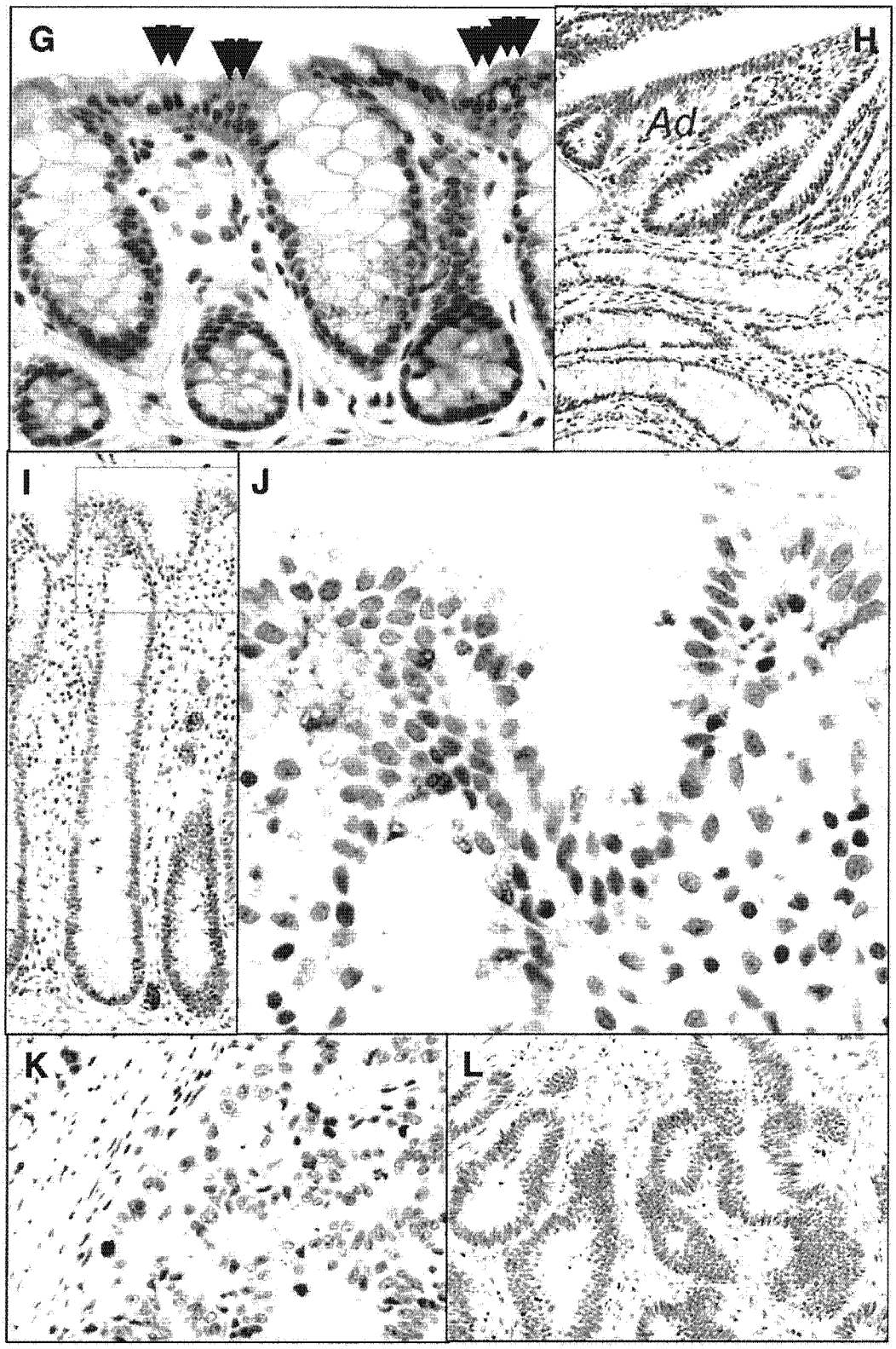


Figure 4. (Cont'd.)

treated total RNA was random primed and reverse transcribed using SuperScript reverse transcriptase (Invitrogen). The following PCR primers were used: for SF1, 5′-CCTTCGGGAAGACGATAACA-3′ and 5′-TTCAGCCATGAGGGACAAAT-3′; for estrogen receptor (ER) β, 5′-CGCTAGAACACACCTTACCTG-3′ and 5′-CTGTGACCAGAGGGTACAT-3′; for WISP1, 5′-CGAGGTACGCAATAGGAGTGTGT-3′ and 5′-CCCTGCCTTAATGAGTGTATGGA-3′; for WISP2, 5′-TTTCTGGCCTTGTCTCTTCC-3′ and 5′-GTGTGTGTAGGCAGGGAGTG-3′; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-AAGGCTGAGAACGGGAAGCTTGTCATCAAT-3′ and 5′-TTCCCGTCTAGCTCAGGGATGACCTTGCCC-3′; for fibroblast growth factor receptor (FGFR) 3, 5′-CGCACCGGCCCATCCTG-3′ and 5′-GCGTCACAGCCGCCACCACC-3′. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining.

Real-Time RT-PCR Analysis

The TaqMan universal PCR master mix and predesigned TaqMan Gene Expression probe and primer sets were purchased from Applied Biosystems. Amplification data measured as an increase in reporter fluorescence were collected in real time with the PRISM 7000 Sequence Detection system (Applied Biosystems). The mRNA expression level of SF1 relative to β-actin was calculated by the comparative threshold cycle (C_T) method.

DNA Sequencing

Automated sequencing was performed using the ABI PRISM Big dye terminator cycle sequencing kit (Applied Biosystems) and a Genetic Analyzer 3100 (Applied Biosystems).

Chemicals

Dox (Sigma-Aldrich), a derivative of tetracycline, was dissolved in deionized water to a stock concentration of 1 mg/mL Dox and added to culture medium to a final concentration of 0.1 μ/mL. Sodium butyrate was obtained from Wako.

Results

Identification of Proteins Whose Expression Is Regulated by the β-Catenin/TCF4 Complex

TCF/LEF lacking the N-terminal β-catenin-binding site suppresses transcriptional activity in a dominant negative manner.[14] By using a strict tetracycline-regulation system,[27] we established a pair of transfectants: DLD1 Tet-off TCF4BΔN30 and DLD1 Tet-off control. DLD1 Tet-off TCF4BΔN30 was capable of inducing TCF4B lacking the NH_2-terminal 30 amino acids (TCF4BΔN30), and DLD1 Tet-off control served as a mock transfectant. Induction of TCF4BΔN30 protein and suppression of the TCF/LEF transcriptional activity in DLD1 Tet-off TCF4BΔN30 cells cultured in the absence of Dox were confirmed by immunoblotting (Figure 1*A*) and

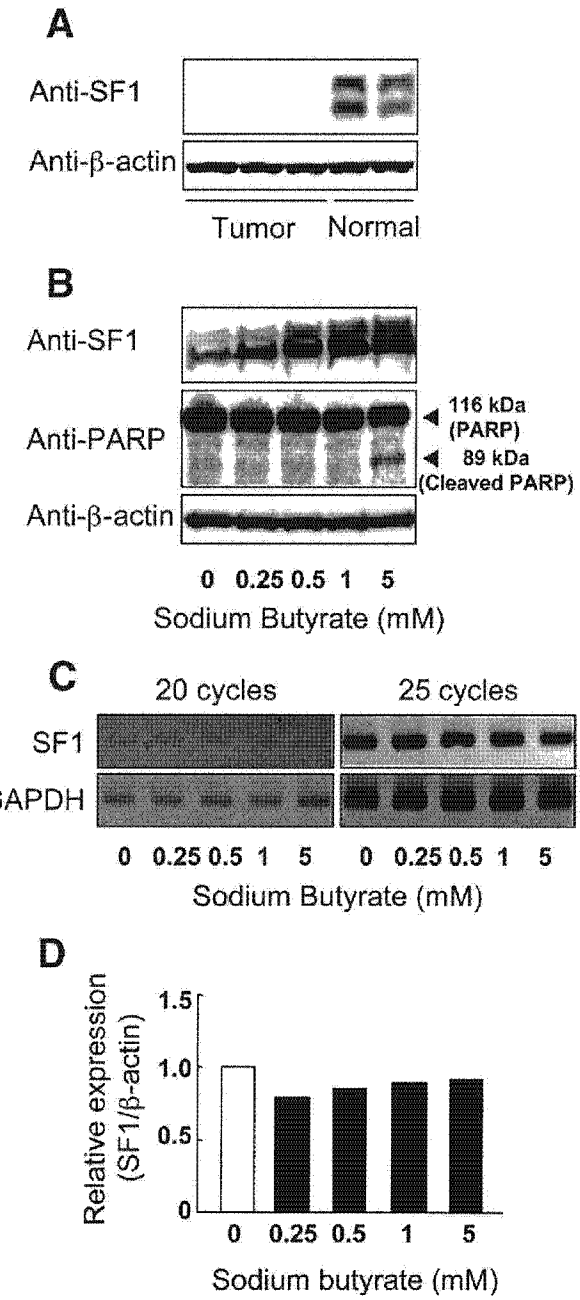


Figure 5. Regulation of SF1 expression by intestinal epithelial cell differentiation. (*A*) Detection of SF1 protein in the tumor (n = 3) and normal (n = 2) small intestine tissue of a Min ($Apc^{Min/+}$) mouse by immunoblotting with anti-SF-1 and anti-β-actin (loading control) antibodies. (*B*) Induction of SF1 protein in DLD1 cells by butyrate. DLD1 cells were incubated for 48 hours without (0) or with sodium butyrate (0.25, 0.5, 1, and 5 mmol/L). Nuclear extracts were analyzed by immunoblotting with anti-SF1, anti-PARP, and anti-β-actin (loading control) antibodies. The detection of the 89-kilodalton cleaved PARP protein indicates induction of apoptosis. (*C* and *D*) Total RNA was extracted from DLD1 cells incubated for 48 hours without (0) or with sodium butyrate (0.25, 0.5, 1, and 5 mmol/L). The expression SF1 and GAPDH (internal control) mRNA was detected by RT-PCR (*I*), and the SF1 level relative to β-actin (ΔC_T) was measured by real-time PCR and expressed as ratios ($\Delta\Delta C_T$) to the control (0) (*J*).

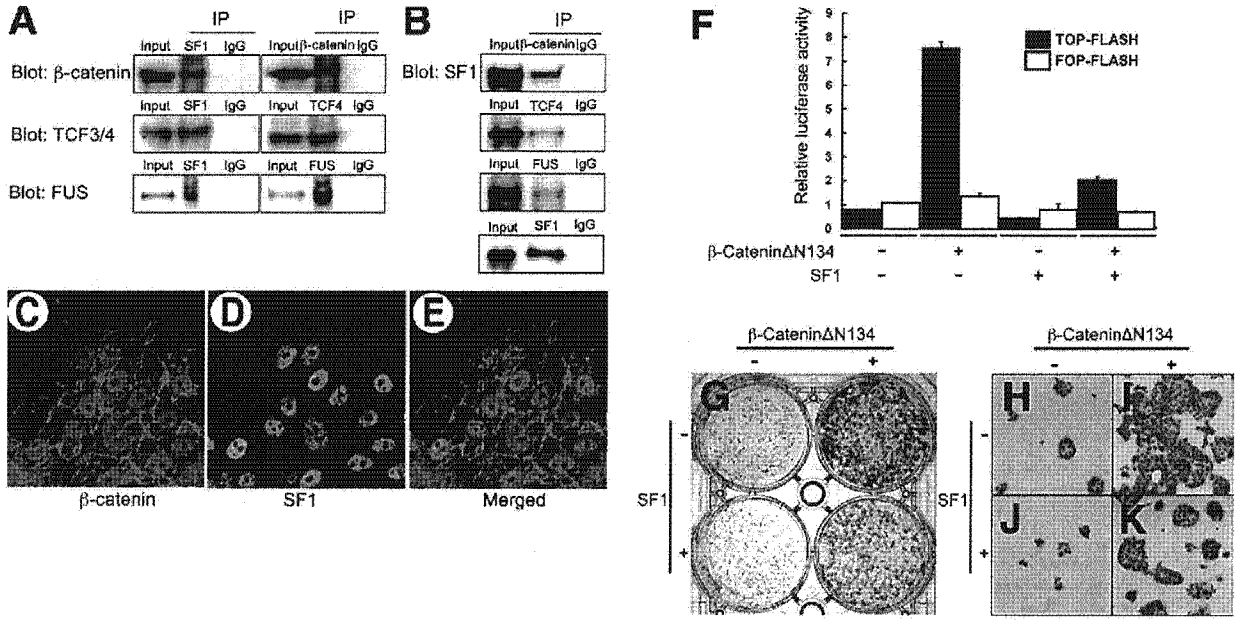


Figure 6. Functional involvement of SF1 in the β-catenin/TCF4 transcriptional complex. (*A* and *B*) DLD1 cells were incubated with 1 mmol/L of sodium butyrate for 48 hours. The nuclear extract was immunoprecipitated (IP) with anti-β-catenin mouse monoclonal antibody, anti-TCF4 mouse monoclonal antibody, control mouse IgG, anti-FUS rabbit polyclonal antibody, control rabbit IgG, anti-SF1 goat polyclonal antibody, or control goat IgG; and the immunoprecipitates were blotted (*Blot*) with anti-SF1, anti-β-catenin, anti-TCF-3/4, and anti-FUS antibodies. (*C–E*) Immunofluorescence staining of β-catenin (*red* in *C* and *E*) and SF1 (*green* in *D* and *E*) proteins in DLD1 cells exposed to sodium butyrate. *E* is a merged image of *C* and *D*. (*F*) HEK293 cells were cotransfected in triplicate with canonical (TOP-FLASH, *solid columns*) or mutant (FOP-FLASH, *open columns*) TCF/LEF luciferase reporters, pFLAG-β-cateninΔN134 (+) or its relevant empty plasmid (pFLAG-CMV4) (−), and pcDNA3.1-His-SF1 (+) or its relevant empty plasmid (pcDNA3.1-His) (−). Luciferase activity was measured 24 hours after transfection. *Bars*, SD. (*G–K*) Colony formation of HEK293 cells transfected with pFLAG-β-cateninΔN134 (+) or its relevant empty plasmid (pFLAG-CMV4) (−) and pcDNA3.1-His-SF1 (+) or its relevant empty plasmid (pcDNA3.1-His) (−). Transfectants were cultured in the presence of G418 for 8 days and stained. Macroscopic (*G*) and microscopic (*H–K*) views are shown.

reporter luciferase assay (Figure 1*B*). The formation of piled-up foci[25] (Figure 1*C*) and colonies in soft agar[25] (Figure 1*D* and 1*E*) was significantly suppressed by the induction of TCF4BΔN30. DLD1 Tet-off TCF4BΔN30 cells and DLD1 Tet-off control cells were cultured for 7 days in the presence and absence of Dox. Whole protein extracts were fractionated into F1 to F5 by stepwise centrifugation (see Supplementary Figure S1 online at www.gastrojournal.org), and the overall protein content was confirmed to be equal by Bradford protein assay (data not shown) and immunoblotting with antibodies against organelle proteins representative of each fraction (see Supplementary Figure S2 online at www.gastrojournal.org).

We used ICAT-MS[28] to search for proteins differentially expressed in DLD1 Tet-off TCF4BΔN30 after induction of dominant negative TCF4 (Figure 2*A* and 2*B*). Whole proteins are enzymatically digested into a large array of small peptide fragments having uniform physical and chemical characteristics. The use of nano (nL/minute)-level flow rate high-performance liquid chromatography (HPLC) coupled with small reverse-phase columns significantly increased the sensitivity of electrospray ionization (ESI)-MS. Among the 4115 and 4441 peptides detected and sequenced in 2 independent ICAT-MS experiments (experiment 1 and experiment 2), 148 (57 up-regulated and 91 down-regulated) and 151 (62 up-regulated and 89 down-regulated) peptides, respectively, were found to be differentially expressed more than 2-fold (see Supplementary Table S1 online at www.gastrojournal.org). Tandem mass spectrometry (MS/MS) (Figure 2*B*) and a database search revealed the 148 and 151 peptides to be derived from 81 (31 up-regulated and 50 down-regulated) and 72 (30 up-regulated and 42 down-regulated) individual proteins, respectively, with 66 overlapping (see Supplementary Table S1 online at www.gastrojournal.org). Based on the functional annotation provided by the Celera Discovery System (CDS) (http://www.celeradiscoverysystem.com/index.cfm), all 87 (= 81 + 72 − 66) proteins compiled from the 2 experiments were classified into 14 (16%) nucleic acid-binding proteins, 7 (8%) oxidoreductases, 7 (8%) select regulatory molecules, 6 (7%) cytoskeletal proteins, and others (see Supplementary Figure S3 online at www.gastrojournal.org). The known subcellular localization of proteins identified by ICAT-MS was generally consistent with results of the organelle fractionation (see Supplementary Table

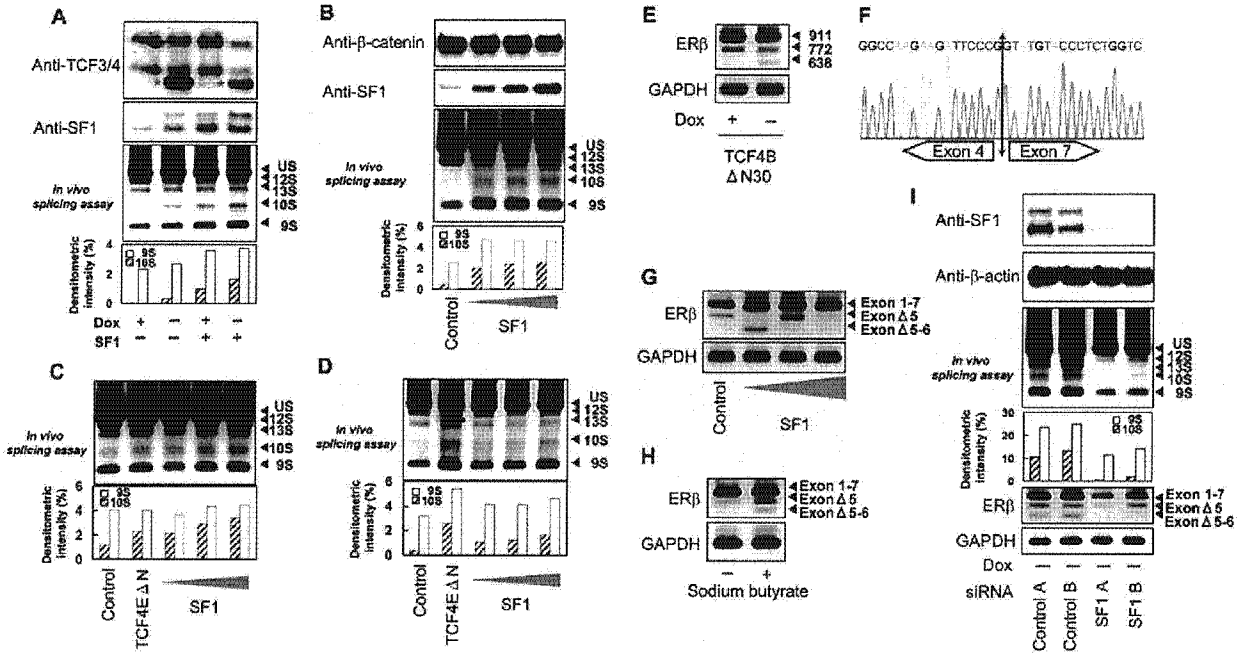


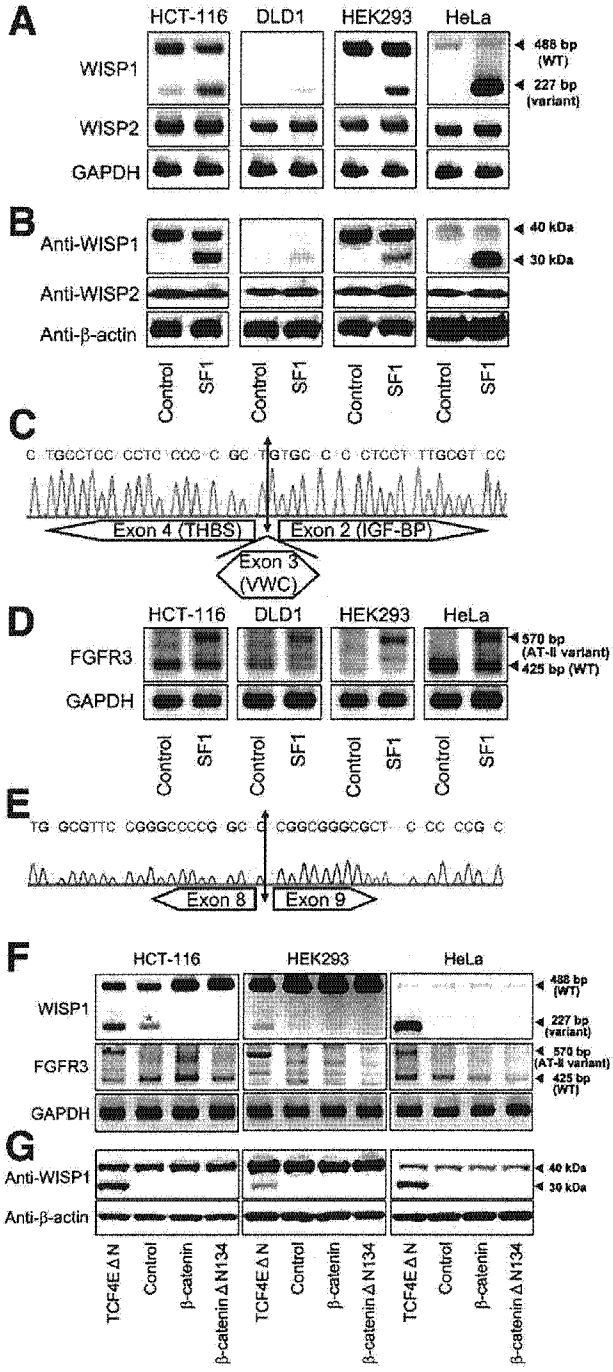
Figure 7. Involvement of SF1 in β-catenin/TCF4-mediated pre-mRNA splicing. (*A*) DLD1 Tet-off TCF4BΔN30 cells were cultured for 5 days in the presence (+) or absence (−) of Dox. The cells were then transiently cotransfected with 0.25 μg pCS3-MT-E1A (adenovirus E1A minigene) and pcDNA3.1-His-SF1 or control pcDNA3.1-His, and an in vivo splicing assay was performed 48 hours after transfection. The relative expression levels of 9S and 10S isoforms are expressed as percentages of the sum of all isoforms. The expression levels of TCF4BΔN30 and SF1 proteins in nuclear extracts of the transfected cells were measured by immunoblotting with anti-TCF3/4 and anti-SF1 antibodies. (*B*) Cos-7 cells were transiently cotransfected with 0.25 μg pCS3-MT-E1A and 0.5, 1.0, or 1.5 μg pcDNA3.1-His-SF1 or pcDNA3.1-His (control) as indicated at the *bottom*, and an in vivo splicing assay was performed 48 hours after transfection. Levels of β-catenin and SF1 protein expression in nuclear extracts of the transfected cells were analyzed by immunoblotting with anti-β-catenin and anti-SF1 antibodies. (*C* and *D*) DLD1 (*C*) and HCT-116 (*D*) cells were transiently cotransfected with 0.25 μg pCS3-MT-E1A and 0.75 μg pFLAG-TCF4EΔN30 or 0.5, 1.0, or 1.5 μg pcDNA3.1-His-SF1 as indicated at the *bottom*. The total amount of DNA used for transfection was kept constant by adding empty plasmid DNA. Total RNA was extracted 48 hours after transfection and analyzed by RT-PCR. (*E*) DLD1 Tet-off TCF4BΔN30 cells were cultured for 7 days in the presence or absence of Dox. Total RNA was extracted, and ERβ and GAPDH (control) mRNA expression were analyzed by RT-PCR. (*F*) DNA sequencing revealed that the 638-bp ERβ splice variant (*E*) lacked exons 5 and 6 (*Exon Δ5-6*). (*G*) HeLa cells were transfected with pcDNA3.1 (control) or 0.5, 1.0, or 1.5 μg pcDNA3.1-His-SF1. The total amount of DNA used for the transfection was kept constant by adding empty plasmid DNA. RT-PCR revealed that SF1 induced expression of splice variants lacking exons 5 and 6 (772 bp, ERβΔ5-6) or exon 5 (638 bp, ERβΔ5), depending on the amount of SF1. (*H*) DLD1 cells were cultured for 48 hours in the absence (−) or presence (+) of 1 mmol/L sodium butyrate. Total RNA was extracted, and expression of ERβ and GAPDH (control) mRNA expression was analyzed by RT-PCR. (*I*) DLD1 Tet-off TCF4BΔN30 cells were cultured for 5 days in the absence of Dox (Dox [−]). The cells were then transiently cotransfected with 0.25 μg pCS3-MT-E1A and SF1 siRNA (SF1 A or SF1 B) or control siRNA (control A or control B). The level of SF1 and β-actin (loading control) protein expression was analyzed by immunoblotting nuclear extracts with anti-SF1 and anti-β-actin antibodies, and the splice pattern of the E1A minigene (in vivo splicing assay) and endogenous ERβ gene was analyzed by RT-PCR.

S2 online at www.gastrojournal.org). We previously reported that 22 out of >2000 protein spots displayed by 2-dimensional difference gel electrophoresis were found to be up- or down-regulated after induction of stabilized β-catenin (β-cateninΔN89),[29] and 9 of the 22 proteins were found to be involved in redox and cytoskeletal regulation.[29] Consistent with those findings, the majority of proteins identified in the current study fell into 2 categories: redox-regulatory proteins and cytoskeleton-associated proteins. Differential expression of representative proteins identified by ICAT-MS was confirmed by immunoblotting with available antibodies (Figure 2*C*). Supplementary Table S2 (see Supplementary Table S2 online at www.gastrojournal.org) lists the proteins identified in this study.

Regulation of SF1 Protein Expression by the β-Catenin/TCF4 Complex

A zinc finger protein, SF1/ZNF162/ZFM1,[30] was identified by ICAT-MS as one of the proteins whose expression was significantly up-regulated upon induction of dominant-negative TCF4B (Figures 2 and 3). DLD1 Tet-off TCF4BΔN30 cells, but not DLD1 Tet-off control cells, increased expression of SF1 protein according to the same time course as induction of TCF4BΔN30 (Figure 3*B*). HEK293 cells transiently transfected with full-length β-catenin (β-catenin) decreased expression of SF1 protein compared with control transfected cells (*, Figure 3*E*). Transient transfection with stabilized β-catenin by deletion of the N-terminal GSK3β-phosphorylation site

(β-cateninΔN134)[15] further decreased the expression of SF1 protein (**, Figure 3*E*), but SF1 mRNA expression was not significantly affected by the induction of dominant negative TCF4B (Figure 3*C* and *D*) or by the β-catenin transfection (Figure 3*F* and *G*). These results suggested that SF1 expression was regulated by the β-catenin/TCF4 complex at the posttranscriptional level.



Regulation of SF1 Expression by Intestinal Epithelial Cell Differentiation

The β-catenin/TCF4 transcription factor complex has been implicated in maintenance of the undifferentiated status of intestinal epithelial cells. Mice lacking TCF4 have been found to have no proliferating compartments in their intestinal crypts.[31] A recent microarray analysis also demonstrated that the TCF4/β-catenin complex switches on genes related to cell proliferation in intestinal epithelial cells and switches off genes related to cell differentiation.[16,32] SF1 is intensely expressed in differentiated epithelial cells of the intestinal villi of multiple intestinal polyposis (Min) ($Apc^{Min/+}$) mice[33] (Figure 4*A* and *B*) but not in undifferentiated cells in the crypts (Figure 4*A* and *C*) or in adenoma cells (Figure 4*C–E*). SF1 was mainly localized in the nuclei of villous cells (and to a lesser degree in their cytoplasm) (Figure 4*B*). Expression of SF1 (Figure 4*E*) varied inversely with accumulation of β-catenin (Figure 4*F*).

Unlike the small intestine, the large intestine does not have a villous-crypt structure and lacks villi. The tubular cryptic cells in the large intestine did not express SF1 protein (Figure 4*G* and *I*), but differentiated epithelial cells lining the luminal surface of the mucosa showed weak nuclear expression of SF1 (Figure 4*G* and *J*). The colon adenoma of a Min mouse lacked expression of SF1 (Figure 4*H*). Only 1 of the 11 sporadic colorectal carcinomas examined showed the nuclear expression of SF1 protein (Figure 4*K*), and the other 10 cases lacked expression (Figure 4*L*). The clinicopathologic significance of SF1 expression in colorectal cancer remains the subject of future study.

Figure 8. Induction of WISP1 and FGFR3 splice variants by SF1. (*A–C*) Induction of WISP1v by SF1. HCT-116, DLD1, HEK293, and HeLa cells were transfected with pcDNA3.1-His (control) and pcDNA3.1-His-SF1; and, 48 hours later, the expression pattern of WISP1, WISP2, and GAPDH mRNA was analyzed by RT-PCR (*A*); and the expression of WISP1, WISP2, and β-actin proteins was analyzed by immunoblotting with anti-WISP1, anti-WISP2, and anti-β-actin antibodies (*B*). DNA sequencing revealed that the 227-bp WISP1 splice variant lacked exon 3 encoding the Von Willebrand type C (VWC) module (*C*). (*D* and *E*) Induction of the FGF3R AT-II splice variant by SF1. HCT-116, DLD1, HEK293, and HeLa cells were transfected with pcDNA3.1-His (control) and pcDNA3.1-His-SF1, and, 48 hours later, the expression pattern of FGF3R and GAPDH mRNA was analyzed by RT-PCR (*D*). DNA sequencing revealed that the 570-bp FGF3R splice variant included both exons 8 and 9 (*E*). THBS, thrombospondin type I domain; IGF-BP, insulin-like growth factor-binding protein-like domain. (*F* and *G*) Induction of the WISP1v and FGF3R AT-II splice variants by dominant negative TCF4. HCT-116, HEK293, and HeLa cells were transfected with empty pFLAG-CMV4, pFLAG-TCF4EΔN30, pFLAG-β-catenin (β-catenin), or pFLAG-β-cateninΔN134 (β-cateninΔN134). The expression pattern of WISP1, FGFR3, and GAPDH mRNA was analyzed by RT-PCR (*F*), and the expression of WISP1 and β-actin proteins was analyzed by immunoblotting with anti-WISP1 and anti-β-actin antibodies (*G*).

Immunoblot analysis confirmed the decreased expression of SF1 protein in the intestinal polyp tissue of the Min mouse compared with normal small intestinal tissue (Figure 5*A*). To confirm further the relationship between expression of SF1 protein and intestinal cell differentiation, DLD1 cells were exposed to sodium butyrate. Butyrate is known to induce cell differentiation, cell cycle arrest, and apoptosis in human colorectal cancer cells.[34] Consistent with the immunohistochemical findings, butyrate increased expression of SF1 protein in a dose-dependent manner (Figure 5*B*). In the 0.25–1 mmol/L range, butyrate was sufficient to induce SF1 but did not cause apoptosis (Figure 5*B*). Butyrate did not significantly affect the level of SF1 mRNA expression (Figure 5*C* and *D*), suggesting that the cell differentiation-associated induction of SF1 is also accomplished at the posttranscriptional level.

We previously identified the FUS oncoprotein[35,36] as a component of the β-catenin/TCF4 complex,[21] and the β-catenin, TCF4, and FUS proteins were coimmunoprecipitated with anti-SF1 antibody but not with normal mouse IgG from a nuclear extract of DLD1 cells exposed to sodium butyrate (Figure 6*A*). Conversely, SF1 protein was coimmunoprecipitated with anti-β-catenin, anti-TCF4, and anti-FUS antibodies (Figure 6*B*), confirming the inclusion of SF1 in the native β-catenin/TCF4/FUS complex. The SF1 expression level was low in DLD1 cells untreated with butyrate (Figure 5*B*), and SF1 was not coimmunoprecipitated with the β-catenin/TCF4 complex (data not shown), suggesting that the interaction is regulated in a cell differentiation-dependent manner. Immunofluorescence microscopy revealed that β-catenin accumulated in the nucleus, cytoplasm, and cell membrane of butyrate-treated DLD1 cells (*red*, Figure 6*C*). SF1 protein was present exclusively in the nuclei (*green*, Figure 6*D*), and its distribution was identical to that of nuclear β-catenin (Figure 6*E*).

Functional Involvement of SF1 in the β-Catenin/TCF4 Transcriptional Complex

Because SF1 has been reported to interact with the NH_2-terminal transcriptional activation domain of EWS and functions as a transcriptional repressor,[23] we investigated the effect of SF1 on the transcriptional activity of the β-catenin/TCF4 complex. We transiently transfected HEK293 cells with β-cateninΔN134 in the presence or absence of SF1 and then measured TCF/LEF reporter activity (Figure 6*F*). Transfection with β-cateninΔN134 (β-cateninΔN134 [+] SF1 [−]) increased the luciferase activity of the canonical TCF/LEF reporter TOP-FLASH[14] (Figure 6*F*, *solid columns*) over mock transfection (β-cateninΔN134 [−] SF1 [−]) but did not increase the luciferase activity of the mutant reporter FOP-FLASH[14] (Figure 6*F*, *open columns*). Cotransfection with SF1 and β-cateninΔN134 suppressed the luciferase activity (β-cateninΔN134 [+] SF1 [+]) in comparison with transfection with β-cateninΔN134 alone (β-cateninΔN134 [+] SF1 [−]).

SF1 transfection markedly suppressed β-cateninΔN134-evoked colony formation by HEK293 cells as measured by a colony formation assay (Figure 6*G–K*), and the colonies formed by the SF1 and β-cateninΔN134 cotransfectants were significantly smaller (Figure 6*K*, β-cateninΔN134 [+] SF1 [+]) than those formed by the β-cateninΔN134 transfectants (Figure 6*I*, β-cateninΔN134 [+] SF1 [−]). A similar inhibitory effect of SF1 on cell growth was observed in colorectal cancer HCT-116 cells (data not shown).

Involvement of SF1 in β-Catenin/TCF4-Mediated Pre-mRNA Splicing

In addition to functioning as a transcriptional repressor, SF1 functions as a regulator of the pre-mRNA splicing reaction. SF1 specifically recognizes the intron branch point sequence, UACUAAC, of nascent pre-mRNA transcripts[37] and is essential for the formation of the functional 17S U2 small nuclear ribonucleoprotein (snRNP) and prespliceosome assembly. We previously reported that β-catenin transfection induces alternations in pre-mRNA splicing,[21] but the precise molecular mechanism underlying it remained undetermined. SF1 seemed to be a good candidate for the effector molecule of β-catenin-mediated alternative pre-mRNA splicing. The adenovirus E1A splicing reporter minigene[26] generated 3 mRNA isoforms (13S, 12S, and 9S) when transfected into DLD1 Tet-off TCF4BΔN30 cells in the presence of Dox (Dox [+], SF1 [−], Figure 7*A*). Removal of Dox induced expression of dominant negative TCF4 (*, *Top*, Figure 7*A*) and increased expression of the 9S and 10S isoforms (Figure 7*A*, Dox [−], SF1 [−]). Transfection with SF1 into DLD1 Tet-off TCF4BΔN30 cells enhanced expression of the 9S and 10S isoforms (Figure 7*A*, Dox [−] and [+], SF1 [+]). SF1 transfection into Cos-7 cells induced expression of the 9S and 10S isoforms in a dose-dependent manner (Figure 7*B*). Similarly, transient transfection of TCF4EΔN30 or SF1 cDNA into DLD1 (Figure 7*C*) or HCT-116 (Figure 7*D*) cells increased the expression of the 9S and 10S isoforms.

We previously reported that β-catenin induces a splicing variant of ERβ lacking exons 5 and 6 (ERβΔ5-6)[21] As a result of the 91 amino acid deletion in the ligand-binding domain, ERβΔ5-6 suppressed the transcriptional activity of wild-type ERβ in a dominant negative manner. RT-PCR revealed that DLD1 Tet-off TCF4BΔN30 cells induced expression of a 638-bp ERβ variant after the removal of Dox (Figure 7*E*), and sequence analysis revealed this variant to be derived from ERβΔ5-6 (Figure 7*F*). The transfection of SF1 into HeLa cells induced expression of a splice variant lacking exons 5 and 6 (ERβΔ5-6) or exon 5 (ERβΔ5), depending on the amount of SF1 (Figure 7*G*). Exposure to butyrate, which promoted differentiation[34] and increased expression of SF1

in DLD1 cells (Figure 5*B*), induced expression of ERβΔ5-6 (Figure 7*H*).

Next, we knocked down SF1 in DLD1 Tet-off TCF4BΔN30 cells expressing dominant negative TCF4 (Dox [−]) by RNA interference (Figure 7*I*). Transfection with 2 different small interfering RNAs (siRNAs) (SF1A and SF1B), but not with 2 control siRNAs (control A and control B), suppressed the expression of SF1 (anti-SF1, Figure 7*I*), and the knockdown of SF1 suppressed the expression of the E1A minigene-derived 9S, 10S, and 13S isoforms ERβΔ5 and ERβΔ5-6 (Figure 7*I*).

Induction of WISP1 and FGFR3 Splice Variants by SF1

WISP1v, a splice variant of the connective tissue growth factor, cysteine-rich-61, nephroblastoma overexpressed-family member WISP1, was originally identified as overexpressed in scirrhous gastric carcinomas.[38] WISP1v, but not wild-type WISP1, induces a striking cellular transformation, rapid piling-up growth, and invasion.[38] WISP1v was induced by transfection of SF1 cDNA into HCT-116, DLD1, HEK293, and HeLa cells (Figure 8*A*), and 30-kilodalton WISP1v protein was detected in the SF1 transfectants by immunoblotting with anti-WISP1 antibody but not in the control transfectants (Figure 8*B*). Sequence analysis confirmed the variant to be derived from an alternative splice variant lacking the Von Willebrand type C module (Figure 8*C*).

FGFR3 normally exists in 2 forms, the IIIb form and the IIIc form, which arise from alternative choice of either exon 8 (FGFR3IIIc) or exon 9 (FGFR3IIIb).[39] In human colorectal cancers, FGFR3 is frequently inactivated by expression of an aberrant splice variant, AT-II, induced by activation of cryptic splice donor sites within exon 7.[39] A 570-bp variant of FGFR3 mRNA was expressed in the SF1 transfectants, but not in the control transfectant (Figure 8*D*), and sequence analysis revealed that the variant was derived from inclusion of both exons 8 and 9 (AT-II) (Figure 8*E*).

The WISP1v and FGF3R AT-II splice variants were also induced by the transient transfection of dominant negative TCF4 (Figure 8*F* and *G*). Conversely, the weak expression of the WISP1v splice variant in HCT-116 cells (*, Figure 8*F*; undetectable by immunoblotting, Figure 8*G*) was inhibited by the transfection of β-catenin or β-cateninΔN134.

Discussion

Genetic and epigenetic alterations during the course of multistage colorectal carcinogenesis, including promoter hypermethylation, loss of heterozygosity, microsatellite/chromosomal instability, and mutation, have been studied extensively,[9,40–43] but posttranslational mechanisms, such as protein expression, protein modifications (glycosylation, ubiquitination, phosphorylation, and others), proteolysis/stabilization, protein-protein interaction, and intra-/intercellular protein transport, also play important roles in colorectal carcinogenesis. In the present study, we carried out a large scale proteomic analysis of colorectal carcinogenesis using a well-characterized cell culture model.[16,19,25] The N-terminally truncated TCF4 protein does not bind β-catenin and acts as an inhibitor of the endogenous β-catenin/TCF complex present in colorectal cancer cells. We examined the expression level of more than 4000 peptides derived from the whole proteome of colorectal cancer cells and identified 87 proteins that were differentially expressed following the induction of dominant negative TCF4 (see Supplementary Table S1 online at www.gastrojournal.org). Because the sensitivity of current proteomic technologies may be still lower than that of cDNA/oligonucleotide microarrays, we were unable to detect the products of known target genes of β-catenin/TCF4, such as c-Myc, CD44, and MDR1.[16,44] However, the whole picture of protein expression profiles altered by inhibiting the β-catenin/TCF complex was well overviewed (see Supplementary Figure S3 online at www.gastrojournal.org).

SF1 was identified as one of the proteins whose expression was negatively regulated by the β-catenin/TCF4 complex (Figure 3). SF1 protein was not detected in undifferentiated intestinal crypt cells or adenoma cells, although it was intensely expressed in differentiated villous cells (Figure 4*A–F*), suggesting that its expression is regulated by the differentiation status of the intestinal epithelium. Consistent with this, the SF1 protein was strongly induced in colon carcinoma cells that had been exposed to a differentiation inducer, sodium butyrate (Figure 5*B*). The public Serial Analysis of Gene Expression (SAGE) data also show decreased expression of SF1 mRNA in colorectal cancer tissue (http://cgap.nci.nih.gov/SAGE/AnatomicViewer). However, the precise molecular mechanism underlying the differentiation-associated regulation of SF1 expression was not determined in this study.

SF1 transfection induced marked suppression of the β-cateninΔN134-evoked TCF/LEF transcriptional activity (Figure 6*F*) and proliferation (Figure 6*G–K*) by HEK293 cells. Consistent with this, SF1 transfection into mouse M1 myeloid leukemia cells has been reported to enhance p53-induced apoptosis,[45] and cytokine-induced down-regulation of SF1 protein has been found to promote rat smooth muscle cell proliferation.[46] Although dominant negative TCF4 (TCF4BΔN30) suppressed the formation of piled-up foci and formation of colonies in soft agar (Figure 1*C–E*), no cell proliferation inhibitory effect was observed under regular tissue culture conditions, probably because the level of endogenous SF1 up-regulation by induction of TCF4BΔN30 was significantly lower than the expression level achieved by transient SF1 cDNA transfection.

Undifferentiated intestinal crypt cells physiologically accumulated the β-catenin protein and expressed the growth-promoting target genes of TCF4.[16] How-

ever, the molecular switch during the physiologic process of epithelial cell differentiation that shuts down the transcriptional activity is unknown. The induction of SF1 protein in intestinal villous epithelial cells (Figure 4*B*) may reflect a differentiation-associated program that prevents the continuity of the β-catenin/TCF4 transcriptional activity. SF-1 was ubiquitously expressed in various organs/tissues of a 17.5-day mouse embryo, and SF1-knockout (*Zfp162*$^{-/-}$) mice died before birth (data not shown), suggesting its involvement in various biologic aspects of embryonic development.

After complete sequencing of the human genome had been accomplished, the number of human protein-encoding genes was estimated to be only 20,000–25,000.[47] However, alternative pre-mRNA splicing results in a generation of multiple transcripts from a single gene and increases the diversity of protein repertoires and cell functions. Human cancers express a large number of alternatively spliced transcripts.[22,48,49] Some of them are known to be generated by somatic and germ-line mutations in exon/intron boundary sequences,[50] but the other molecular mechanisms causing cancer-related splice aberrations are largely unknown.

In this study, we demonstrated that knockdown of SF1 with 2 different siRNAs compromised the induction of alternative splicing of the adenovirus E1A minigene and the ERβ gene by dominant negative TCF4 (Figure 7*I*). However, the regulation of pre-mRNA splicing seems to be very complicated. SF1 expression was associated with intestinal epithelial cell differentiation, yet SF1 was also responsible for the induction of known cancer-related splice variants, such as WISP1v[38] and FGF3R-ATII[39] (Figure 8*A–E*). The level of SF1 protein expression is regulated by the β-catenin/TCF4 complex (Figure 3); however, SF1 affects the gene transactivation (Figure 6*F*) and the pre-mRNA splicing (Figure 7) activities of the β-catenin/TCF4 complex. The expression pattern of ERβ splice variants changed significantly according to the amount of SF1 (Figure 7*G*). It will be necessary to investigate how β-catenin, TCF4, FUS, SF1, and other RNA-binding proteins in the complex influence the mutual mRNA/protein expression levels and how these proteins regulate the selection of exons and relative/absolute expression levels of different variants.

Alternatively spliced RNA variants with tumor specificity may be good candidate cancer biomarkers and therapy targets.[51] Also, aberrant splice variants expressed in human cancers may have functional properties that are distinct from their wild-type counterparts[49,52] and be causatively involved in the initiation and progression of carcinogenesis. A large scale oligonucleotide microarray that covers all the exons of the human genome has been developed (http://www.affymetrix.com). This new type of technology may reveal the whole picture of splice variations induced by the β-catenin/Wnt signaling pathway and expand our knowledge of how dysregulation of transcription and splicing affects the multigene network of carcinogenesis.

β-Catenin may not initiate intestinal carcinogenesis by transactivation of a limited number of cell proliferation-promoting target genes of TCF4 alone. Our recent series of proteomic studies has revealed various aspects of β-catenin function in the regulation of cell motility,[53] DNA damage recognition,[54] and pre-mRNA splicing.[21] Elucidation of the entire protein network assembled by these pathways may provide new insights for the development of therapeutic strategies for colorectal cancer.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1053/j.gastro.2007.01.007.

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