Ku70 and Poly(ADP-Ribose) Polymerase-1 Competitively Regulate β-Catenin and T-Cell Factor-4–Mediated Gene Transactivation: Possible Linkage of DNA Damage Recognition and Wnt Signaling

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Abstract

Formation of the T-cell factor-4 (TCF-4) and β-catenin nuclear complex is considered crucial to embryonic development and colorectal carcinogenesis. We previously reported that poly(ADP-ribose) polymerase-1 (PARP-1) interacts with the TCF-4 and β-catenin complex and enhances its transcriptional activity. However, its biological significance remains unexplained. Using immunoprecipitation and mass spectrometry, we found that two Ku proteins, Ku70 and Ku80, were also associated with the complex. Knockdown of Ku70 by RNA interference increased the amount of β-catenin associated with TCF-4 and enhanced the transcriptional activity. PARP-1 competed with Ku70 for binding to TCF-4. Treatment with bleomycin, a DNA-damaging alkylating agent, induced polyADP-ribosylation of PARP-1 protein and inhibited its interaction with TCF-4. Bleomycin conversely increased the amounts of Ku70 coimmunoprecipitated with TCF-4 and removed β-catenin from TCF-4. We propose a working model in which the transcriptional activity of TCF-4 is regulated by the relative amount of Ku70, PARP-1, and β-catenin proteins binding to TCF-4. Identification of the functional interaction of Ku70 as well as PARP-1 with the TCF-4 and β-catenin transcriptional complex may provide insights into a novel linkage between DNA damage recognition/repair and Wnt signaling. [Cancer Res 2007;67(3):911–8]

Introduction

The Wnt signaling pathway plays important roles in embryogenesis and carcinogenesis (1). Secreted Wnt molecules bind to cell membrane Frizzled receptors and evoke downstream intracellular signaling. The signal is then transmitted to a multiprotein complex consisting of the *APC* gene product, Axin/Axil, and glycogen synthase kinase 3β (GSK3β), a chaperone that supports the phosphorylation of β-catenin by GSK3β (2, 3). Phosphorylated β-catenin protein is subject to rapid degradation via the ubiquitin-proteasome pathway (4). The Wnt signaling inhibits GSK3β and increases the cytoplasmic β-catenin content. Mutation of either the *APC* or β-catenin (*CTNNB1*) gene is frequently seen in colorectal carcinoma and mimics the constitutively active Wnt signaling (5, 6). The excess β-catenin protein acts as a transcriptional coactivator by forming complexes with T-cell factor (TCF)/lymphoid enhancer factor (LEF) family DNA-binding proteins (7). TCF-4 is a member of the TCF/LEF family commonly expressed in colorectal epithelium and cancer cells (8). TCF-4 has been implicated in the maintenance of undifferentiated intestinal crypt epithelial cells because no proliferative compartments have been detected in the intestinal crypts of mice lacking TCF-4 (9). Constitutive transactivation of the target genes of TCF-4 by accumulation of β-catenin protein imposes a crypt progenitor phenotype on intestinal epithelial cells and is considered crucial to the initiation of colorectal carcinogenesis (10).

In our previous study, we found that poly(ADP-ribose) polymerase-1 (PARP-1) interacted with the TCF-4 and β-catenin nuclear complex (11). PARP-1 was originally identified as a nuclear DNA-binding protein that catalyzes the transfer of ADP-ribose from NAD^+ to acceptor proteins (12). PARP-1 is activated by DNA damage and plays an important role in the process of DNA repair and genomic stability (13).

Besides DNA damage recognition and apoptosis, the role of PARP-1 as a regulator of various transcription factors has recently attracted a great deal of attention (14). We have found that PARP-1 is a component and enhancer of the TCF-4 and β-catenin transcriptional complex (11). PARP-1 polyADP-ribosylates its own automodification domain in response to DNA damage (12). PolyADP-ribosylation of PARP-1 inhibits the interaction with TCF-4 and its transcriptional activity (11). However, the biological significance of the interaction between TCF-4 and PARP-1 and its inhibition by polyADP-ribosylation of PARP-1 remains unexplained.

In this study, we further explored the protein components of the TCF-4 and β-catenin nuclear complex and identified that Ku70 and Ku80 proteins interact with TCF-4. The Ku autoantigen was originally identified as a nuclear protein recognized by autoantibodies in sera of patients with polymyositis-scleroderma overlap syndrome (15). The Ku autoantigen consists of two subunit proteins of ~70 kDa and 80 to 86 kDa (named Ku70 and Ku80). Ku recognizes DNA double strand breaks and then recruits the DNA-dependent protein kinase catalytic subunit (DNA-PKcs; ref. 16). The Ku70/Ku80/DNA-PKcs complex mediates nonhomologous end joining and repairs double strand breaks (17). Ku proteins are also involved in other cellular processes such as immunoglobulin gene rearrangement, telomere maintenance, apoptosis, and transcriptional regulation (18). Here, we report that Ku70 is a novel inhibitor of the β-catenin/TCF-4 transcriptional complex.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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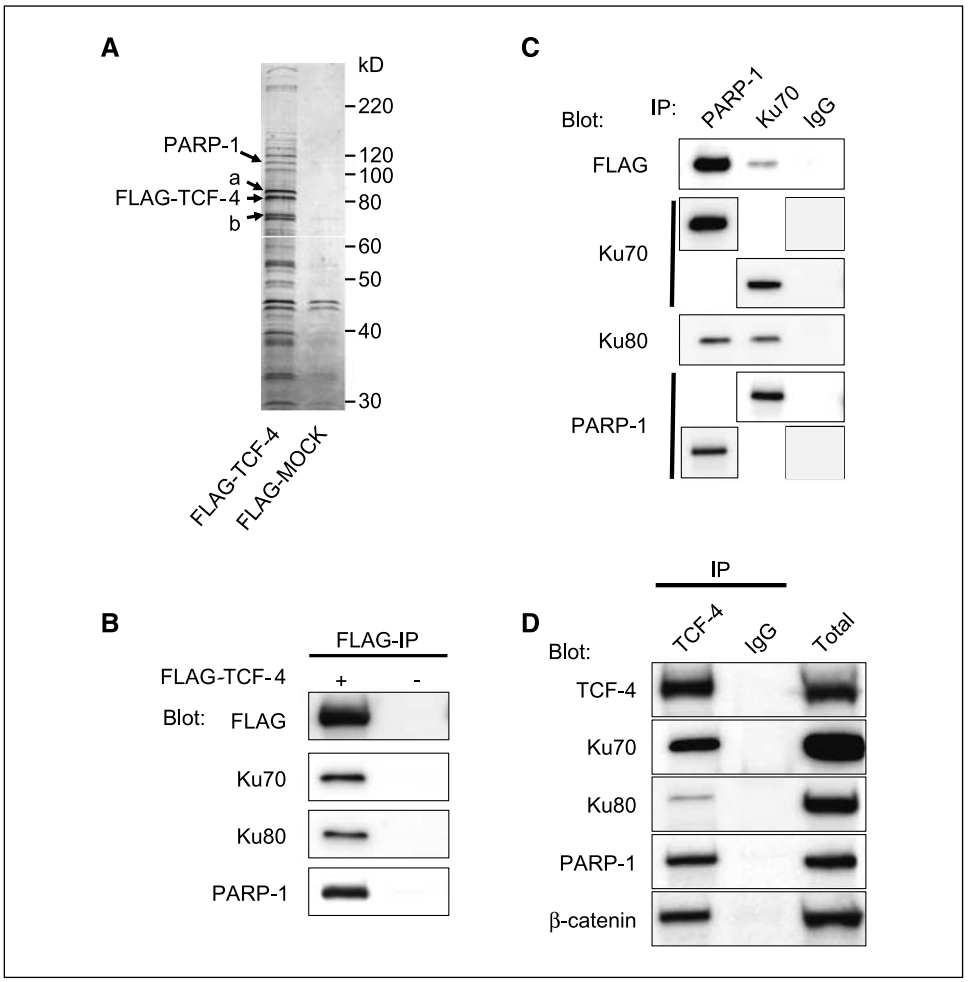

Figure 1. Identification of interaction between Ku and TCF-4. *A,* HEK293 cells were transfected with FLAG–TCF-4 or control FLAG-MOCK. Nuclear extracts were immunoprecipitated with anti-FLAG affinity gel (modified from ref. 11 with permission). *B,* Western blot analysis of the immunoprecipitates (*IP*) of HEK293 cells transfected with FLAG–TCF-4 (+) or control FLAG-MOCK (−). The immunoprecipitates with anti-FLAG affinity beads were blotted with anti-FLAG, anti-Ku70, anti-Ku80, and anti–PARP-1 antibodies. *C,* lysate of HEK293 cells transfected with FLAG–TCF-4 was immunoprecipitated with anti–PARP-1 and anti-Ku70 antibody or normal mouse IgG and blotted with anti-FLAG, anti-Ku70, anti-Ku80, and anti–PARP-1 antibodies. *D,* nuclear extract of HCT116 cells (*Total*) was immunoprecipitated with anti–TCF-4 antibody or normal mouse IgG and blotted with anti–TCF-4, anti-Ku70, anti-Ku80, anti–PARP-1, and anti–β-catenin antibodies.

Materials and Methods

Cell culture. The human embryonal kidney cell line HEK293 was obtained from the Riken Cell Bank (Tsukuba, Japan). Hepatoblastoma cell line HepG2 and colorectal cancer cell lines HCT116, DLD-1, and SW480 were purchased from the American Type Culture Collection (Manassas, VA). The Li7 cell line was established from a patient with hepatocellular carcinoma as reported previously (19). PARP-null mouse embryonic fibroblast (MEF) was established from a PARP-1 knockout (*Parp1*$^{-/-}$) mouse (20).

Cells were treated with 5 mmol/L hydroxyurea (Sigma, St. Louis, MO) for 18 h at 37°C. The medium was then removed, and incubation was continued with serum-free medium without or with bleomycin (50 μg/mL; Sigma).

Plasmid constructs. Human TCF-4 cDNA and its truncated forms were subcloned into pFLAG-CMV4 (Sigma). Human Ku70 cDNA and its truncated forms were subcloned into pcDNA3.1/myc-His (Invitrogen, Carlsbad, CA). Human PARP-1 cDNA (kindly provided by Dr. M. Miwa, Nagahama Institute of Bio-Science and Technology, Nagahama, Japan) was subcloned into pcDNA3.1/myc-His. Human β-cateninΔN134 cDNA was subcloned into pCR3.1 (Invitrogen), which lacks a 134-amino-acid sequence at its NH_2 terminus. The composition of all of the constructs in this study was confirmed by restriction endonuclease digestion and sequencing.

Immunoprecipitation. Cells were extracted with lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton X-100] containing a protease inhibitor cocktail (Sigma). Nuclear extracts were prepared with the CelLytic nuclear extraction kit (Sigma). Immunoprecipitation was done with 50 μL of anti-FLAG M2 affinity gel (Sigma) or anti–PARP-1 monoclonal antibody (BD PharMingen, San Diego, CA), anti-Ku70 (Ab-5) monoclonal antibody (Lab Vision, Fremont, CA), and anti–TCF-4 monoclonal antibody (Upstate, Charlottesville, VA) along with 10 μL of Dynabeads Protein G (Dynal, Oslo, Norway). After being washed with washing buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl], immobilized immunocomplexes were eluted from anti–FLAG M2 affinity gel by incubation at 4°C with 150 ng/μL 3×FLAG Peptide (Sigma) or from Dynabeads by boiling in SDS loading buffer. Proteins were fractionated by SDS-PAGE and detected using a negative gel stain MS kit (Wako, Osaka, Japan) or by Western blotting.

Protein identification by mass spectrometry. SDS-PAGE gels were cut into ~1-mm^3 sections, reduced with NH_4HCO_3, and alkylated with iodoacetamide. The gel sections were then washed with acetonitrile, hydrolyzed with modified trypsin (Promega, Madison, WI), and incubated at 37°C overnight. Peptides eluted from the gel sections were spotted onto a steel target plate along with 2,5-dihydroxybenzoic acid (gentisic acid; Sigma) as a matrix. Mass spectra were obtained in the refractor mode by using a Q-star Pulsar-*i* mass spectrometer (Applied Biosystems, Foster City, CA) and analyzed using Mascot software (Matrix Sciences, London, United Kingdom; ref. 21).

Western blot analysis. Anti–FLAG M2 monoclonal antibody was purchased from Sigma; anti-Ku70 (Ab-4) and anti-Ku80 (Ab-2) monoclonal antibodies were from Lab Vision; anti–β-catenin monoclonal antibody was from BD Transduction (Lexington, KY); anti-TCF3/4 monoclonal antibody was from Upstate; and anti-PARP polyclonal antibody was from Trevigen (Gaithersburg, MD). Total cell lysates were extracted at 4°C with radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mmol/L Tris-HCl (pH 8.0)]. Samples were

fractionated by SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Billerica, MA), and the blots were detected using an enhanced chemiluminescence method (Amersham, Piscataway, NJ).

Reverse transcription-PCR. Total RNA was prepared with an RNeasy mini kit (Qiagen, Valencia, CA), and 1-μg samples of total RNA were reverse transcribed. cDNA samples from tissues of human sporadic colorectal cancer and the corresponding normal tissues were obtained from Clontech (Palo Alto, CA). The PCR products were analyzed by agarose gel electrophoresis. The sequences of all the PCR primers in this study are available upon request.

Luciferase reporter assay. A pair of luciferase reporter constructs, TOP-FLASH and FOP-FLASH (Upstate), were used to evaluate TCF/LEF transcriptional activity. Cells were transiently transfected in triplicate with one of the luciferase reporters and phRG-TK (Promega) using Lipofectamine 2000 reagent (Invitrogen). Luciferase activity was measured with the Dual-luciferase reporter assay system (Promega) and *Renilla* luciferase activity as an internal control.

RNA interference. Two short hairpin RNA (shRNA) sequences targeting Ku70 mRNA were designed by B-Bridge (Sunnyvale, CA). Synthesized double-stranded oligonucleotides were cloned into the pSUPER RNA interference vector (OligoEngine, Seattle, WA) carrying the H1 promoter and neomycin resistance gene.

Immunofluorescence microscopy. Cells were grown on poly-L-lysine–coated coverslips (Asahi Technoglass, Funabashi, Japan). After being fixed with 3.7% paraformaldehyde, the cells were incubated with anti-PARP rabbit polyclonal antibody and anti–PARP-1 mouse monoclonal antibody (BD Transduction) overnight at 4°C. Following incubation with Alexa Fluor 488–labeled goat anti-mouse IgG and Alexa Fluor 594–labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR), the coverslips were inspected with a laser scanning confocal microscope (Bio-Rad, Hercules, CA).

Immunohistochemistry. Ten familial adenomatous polyposis (FAP) patients were selected from the surgical pathology panel of the National Cancer Center Central Hospital. Formalin-fixed and paraffin-embedded intestinal tissues containing adenomas were stained by the avidin-biotin complex method as previously described (22).

Results

Identification of a novel interaction between the TCF-4 and Ku proteins. HEK293 cells were transiently transfected with FLAG-tagged TCF-4 (FLAG–TCF-4) or a control plasmid (FLAG-MOCK). Immunoprecipitation with anti-FLAG antibody and SDS-PAGE revealed that several proteins were selectively coimmunoprecipitated with FLAG–TCF-4, but not with the control (Fig. 1*A*). We had

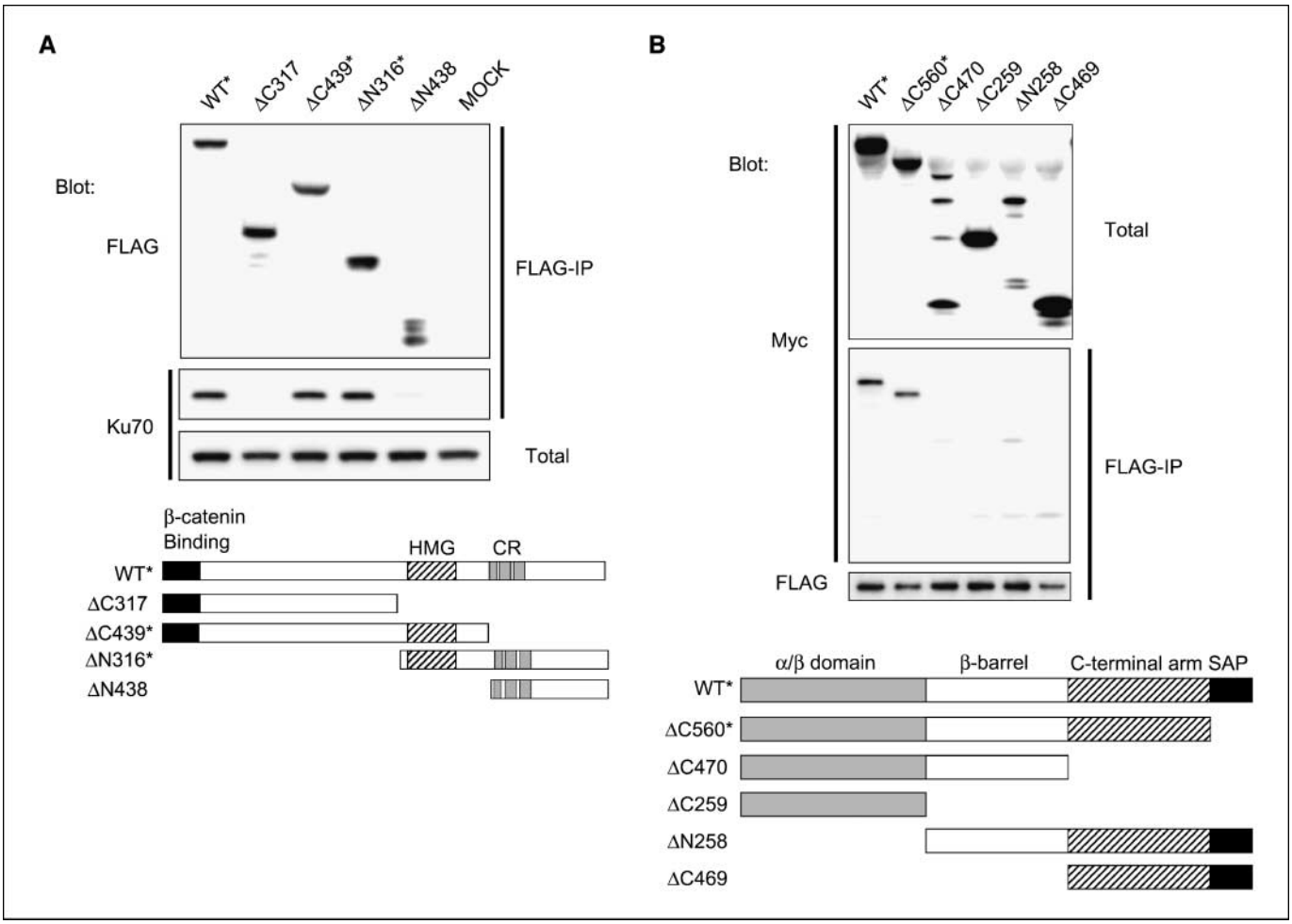

Figure 2. Binding domains necessary for interaction between Ku70 and TCF-4. *A,* full-length or truncated forms of FLAG–TCF-4 were transfected into HEK293 and immunoprecipitated with anti-FLAG affinity beads. The complexes were analyzed by blotting with anti-FLAG and anti-Ku70 antibodies. The full-length and truncated forms of TCF-4 are represented schematically at the bottom. *, TCF-4 constructs that bound to the Ku70 protein. *B,* Myc-tagged full-length or truncated forms of pcDNA3.1-Ku70 and full-length FLAG–TCF-4 were cotransfected into HEK293 and immunoprecipitated with anti-FLAG affinity beads. The complexes were analyzed by blotting with anti-Myc and anti-FLAG antibodies. The full-length and truncated forms of Ku70 are represented schematically at the bottom. *, Ku70 constructs that bound to the TCF-4 protein.

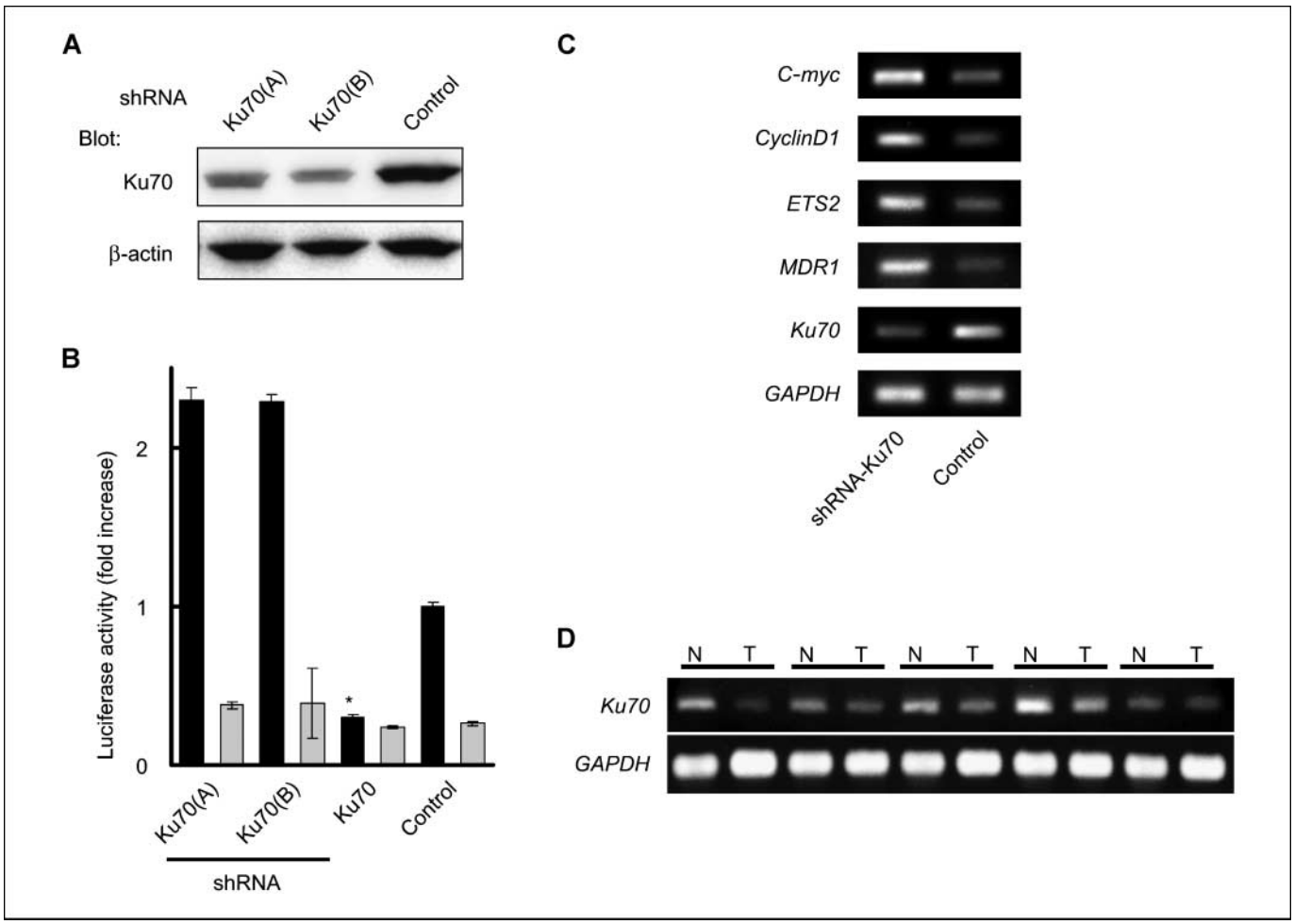

Figure 3. Ku70 suppresses gene transcriptional activity of TCF-4. *A,* Western blot analysis showing the protein level of Ku70 (*top*) and β-actin (loading control, *bottom*) of HCT116 cells transfected with pSUPER-Ku70 [*Ku70(A), Ku70(B)*] or pSUPER-control (*Control*). *B*, HCT116 cells were cotransfected with pSUPER-Ku70(A), pSUPER-Ku70(B), pcDNA3.1-Ku70 (*Ku70*), or control plasmid as well as canonical (TOP-FLASH) or mutant (FOP-FLASH) TCF/LEF 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 and expressed as a fold increase. *C,* HCT116 cells were transiently transfected with a mixture of pSUPER-Ku70(A) and pSUPER-Ku70(B) (*shRNA-Ku70*) or empty pSUPER (*Control*). Forty-eight hours after transfection, the expression levels of *c-myc, cyclin D1, ETS2, MDR1, Ku70*, and *GAPDH* mRNA were analyzed by reverse transcription-PCR. *D,* expression of Ku70 and GAPDH mRNA in paired samples of normal intestine (*N*) and cancer (*T*) tissues from five patients with sporadic colorectal cancer.

previously identified one of these proteins as PARP-1 (Fig. 1*A*). Proteins of ~70 kDa (Fig. 1*A, b*) and 86 kDa (Fig. 1*A, a*) were also constantly coimmunoprecipitated with FLAG-tagged TCF-4 and were subjected to protein identification by mass spectrometry. Peptide mass fingerprinting and tandem mass spectrometry (data not shown) revealed that these proteins were Ku70 (70-kDa thyroid autoantigen/thyroid-lupus autoantigen/G22P1) and Ku80 (X-ray repair, complementing defective, in Chinese hamster, 5/XRCC5).

The protein identification was confirmed by Western blotting with anti-Ku70 and anti-Ku80 antibodies. Ku70, Ku80, and PARP-1 proteins were detected in the immunoprecipitate with anti-FLAG antibody (Fig. 1*B*). Ku70, Ku80, and FLAG-tagged TCF-4 proteins were detected in the immunoprecipitate with anti–PARP-1 antibody (Fig. 1*C, IP: PARP-1*). FLAG-tagged TCF-4 (FLAG), Ku80, and PARP-1 were also detected in the immunoprecipitate with anti-Ku70 antibody (Fig. 1*C, IP: Ku70*) but not with control mouse IgG (Fig. 1*C, IP: IgG*).

Ku70, Ku80, PARP-1, and β-catenin proteins were coimmunoprecipitated with endogenous TCF-4 from a lysate of colorectal cancer HCT116 cells (Fig. 1*D*). Ku70 and Ku80 were also coimmunoprecipitated with PARP-1 (Supplementary Fig. S1), suggesting that Ku70, Ku80, and PARP-1 are native components of the TCF-4 and β-catenin complex.

Binding domains necessary for the interaction between Ku70 and TCF-4. To identify the region of TCF-4 that is essential for its interaction with Ku70, we expressed serially truncated forms of FLAG–TCF-4 and evaluated their binding activity to Ku70 (Fig. 2*A*). Only constructs carrying the high-mobility group (HMG) box [wild-type (WT), ΔC439, and ΔN316] were found to bind to Ku70 (Fig. 2*A*).

The Ku70 protein consists of four domains: the α/β domain, β-barrel, COOH-terminal arm, and scaffold attachment factor (SAP) DNA-binding domain (23). We evaluated the ability of Ku70 serially truncated at the border of each domain to bind to FLAG–TCF-4 (Fig. 2*B*). Only the full-length Ku70 protein (WT) and the Ku70 protein lacking the SAP domain (ΔC560) interacted with TCF-4 (Fig. 2*B*). These results suggest that the three-dimensional structure of Ku70 protein rather than the specific amino acid

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

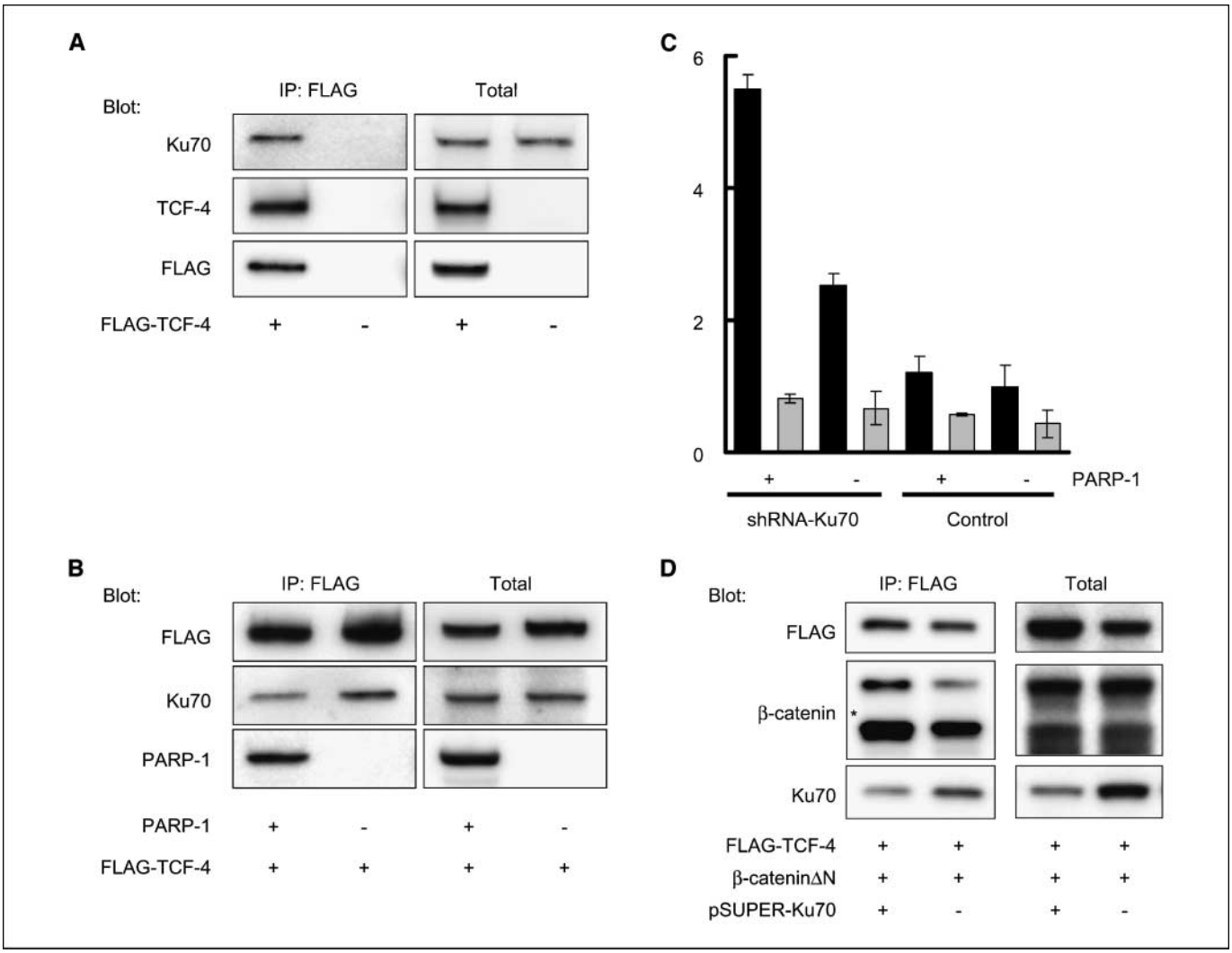

Figure 4. Competitive regulation of the TCF-4 and β-catenin complex by Ku70 and PARP-1. *A,* PARP-1–null MEF were transfected with FLAG–TCF-4 or FLAG-MOCK, and whole lysates (*Total*) and immunoprecipitates with anti-FLAG affinity beads (*IP: FLAG*) were blotted with anti-Ku70, anti-TCF-4, and anti-FLAG 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.

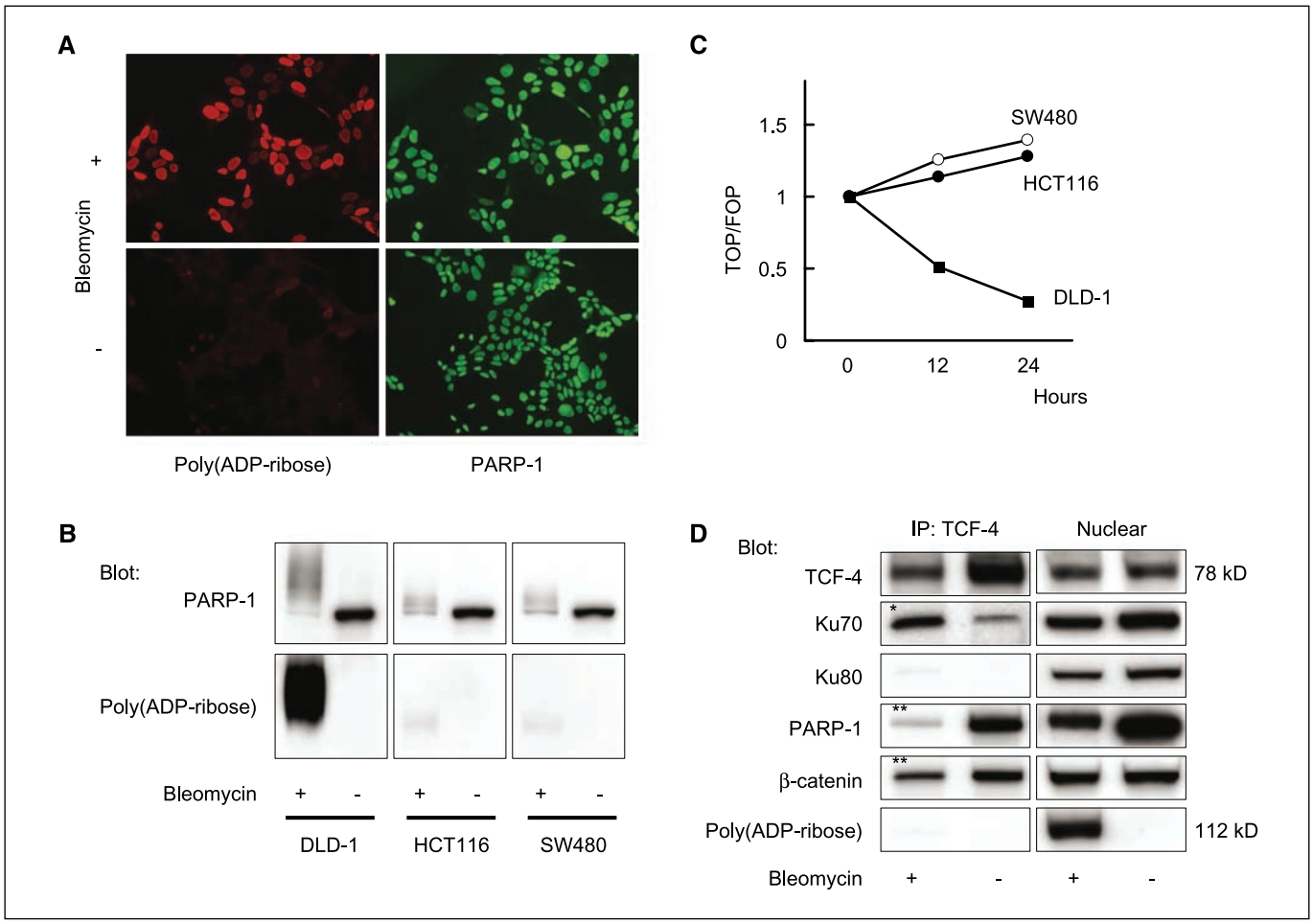

Figure 5. Possible linkage of DNA damage recognition and Wnt signaling. *A,* detection of polyADP-ribosylation in DLD-1 cells incubated with medium containing, or lacking, bleomycin for 6 h. Immunofluorescence staining was done using anti–poly(ADP-ribose) rabbit polyclonal antibody (*red*) and anti–PARP-1 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).

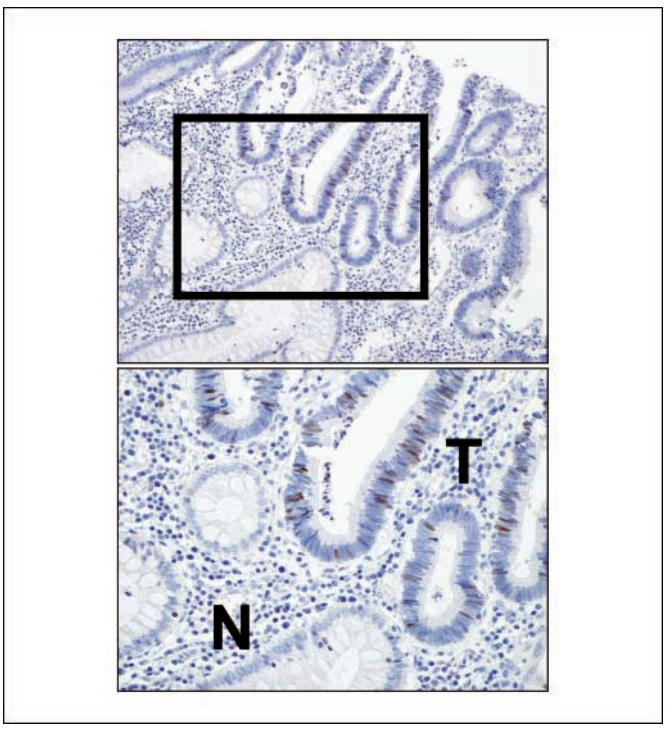

Figure 6. Nuclear poly(ADP-ribose) formation in colorectal adenoma. Immunohistochemistry of colorectal adenoma and normal glands of a FAP patient. Formation of poly(ADP-ribose) was detected with anti–poly(ADP-ribose) polyclonal antibody.

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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References

1. Peifer M, Polakis P. Wnt signaling in oncogenesis and embryogenesis—a look outside the nucleus. Science 2000;287:1606–9.
2. Nelson WJ, Nusse R. Convergence of Wnt, β-catenin, and cadherin pathways. Science 2004;303:1483–7.
3. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med 2004;10:789–99.
4. Aberle H, Bauer A, Stappert J, Kispert A, Kemler R. β-Catenin is a target for the ubiquitin-proteasome pathway. EMBO J 1997;16:3797–804.
5. Munemitsu S, Albert I, Souza B, Rubinfeld B, Polakis P. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor-suppressor protein. Proc Natl Acad Sci U S A 1995;92:3046–50.
6. Fearnhead NS, Britton MP, Bodmer WF. The ABC of APC. Hum Mol Genet 2001;10:721–33.
7. Behrens J, von Kries JP, Kuhl M, et al. Functional interaction of β-catenin with the transcription factor LEF-1. Nature 1996;382:638–42.
8. Korinek V, Barker N, Morin PJ, et al. Constitutive transcriptional activation by a β-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. Science 1997;275:1784–7.
9. Korinek V, Barker N, Moerer P, et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. Nat Genet 1998;19:379–83.
10. van de Wetering M, Sancho E, Verweij C, et al. The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 2002;111:241–50.
11. Idogawa M, Yamada T, Honda K, Sato S, Imai K, Hirohashi S. Poly(ADP-ribose) polymerase-1 is a component of the oncogenic T-cell factor-4/β-catenin complex. Gastroenterology 2005;128:1919–36.
12. de Murcia G, Menissier de Murcia J. Poly(ADP-ribose) polymerase: a molecular nick-sensor. Trends Biochem Sci 1994;19:172–6.
13. Herceg Z, Wang ZQ. Functions of poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. Mutat Res 2001;477:97–110.
14. Kim MY, Mauro S, Gevry N, Lis JT, Kraus WL. NAD^+-dependent modulation of chromatin structure and transcription by nucleosome binding properties of PARP-1. Cell 2004;119:803–14.
15. Mimori T, Akizuki M, Yamagata H, Inada S, Yoshida S, Homma M. Characterization of a high molecular weight acidic nuclear protein recognized by autoantibodies in sera from patients with polymyositis-scleroderma overlap. J Clin Invest 1981;68:611–20.
16. Dynan WS, Yoo S. Interaction of Ku protein and DNA-dependent protein kinase catalytic subunit with nucleic acids. Nucleic Acids Res 1998;26:1551–9.
17. Lieber MR, Ma Y, Pannicke U, Schwarz K. Mechanism and regulation of human non-homologous DNA end-joining. Nat Rev Mol Cell Biol 2003;4:712–20.
18. Downs JA, Jackson SP. A means to a DNA end: the many roles of Ku. Nat Rev Mol Cell Biol 2004;5:367–78.
19. Osada T, Sakamoto M, Ino Y, et al. E-cadherin is involved in the intrahepatic metastasis of hepatocellular carcinoma. Hepatology 1996;24:1460–7.
20. Masutani M, Suzuki H, Kamada N, et al. Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes. Proc Natl Acad Sci U S A 1999;96:2301–4.
21. Sato S, Idogawa M, Honda K, et al. β-catenin interacts with the FUS proto-oncogene product and regulates pre-mRNA splicing. Gastroenterology 2005;129:1225–36.
22. Seike M, Kondo T, Mori Y, et al. Proteomic analysis of intestinal epithelial cells expressing stabilized β-catenin. Cancer Res 2003;63:4641–7.
23. Walker JR, Corpina RA, Goldberg J. Structure of the Ku heterodimer bound to DNA and its implications for double-strand break repair. Nature 2001;412:607–14.
24. Li B, Navarro S, Kasahara N, Comai L. Identification and biochemical characterization of a Werner's syndrome protein complex with Ku70/80 and poly(ADP-ribose) polymerase-1. J Biol Chem 2004;279:13659–67.
25. Rigas B, Borgo S, Elhosseiny A, et al. Decreased expression of DNA-dependent protein kinase, a DNA repair protein, during human colon carcinogenesis. Cancer Res 2001;61:8381–4.
26. Giffin W, Torrance H, Rodda DJ, Prefontaine GG, Pope L, Hache RJ. Sequence-specific DNA binding by Ku autoantigen and its effects on transcription. Nature 1996;380:265–8.
27. Maldonado E, Shiekhattar R, Sheldon M, et al. A human RNA polymerase II complex associated with SRB and DNA-repair proteins. Nature 1996;381:86–9.
28. Kuhn A, Gottlieb TM, Jackson SP, Grummt I. DNA-dependent protein kinase: a potent inhibitor of transcription by RNA polymerase I. Genes Dev 1995;9:193–203.
29. Dvir A, Stein LY, Calore BL, Dynan WS. Purification and characterization of a template-associated protein kinase that phosphorylates RNA polymerase II. J Biol Chem 1993;268:10440–7.
30. Iijima S, Teraoka H, Date T, Tsukada K. DNA-activated protein kinase in Raji Burkitt's lymphoma cells. Phosphorylation of c-Myc oncoprotein. Eur J Biochem 1992;206:595–603.
31. Bannister AJ, Gottlieb TM, Kouzarides T, Jackson SP. c-Jun is phosphorylated by the DNA-dependent protein kinase *in vitro*; definition of the minimal kinase recognition motif. Nucleic Acids Res 1993;21:1289–95.
32. Singleton BK, Torres-Arzayus MI, Rottinghaus ST, Taccioli GE, Jeggo PA. The C terminus of Ku80 activates the DNA-dependent protein kinase catalytic subunit. Mol Cell Biol 1999;19:3267–77.
33. Sucharov CC, Helmke SM, Langer SJ, Perryman MB, Bristow M, Leinwand L. The Ku protein complex interacts with YY1, is up-regulated in human heart failure, and represses α myosin heavy-chain gene expression. Mol Cell Biol 2004;24:8705–15.
34. Galande S, Kohwi-Shigematsu T. Poly(ADP-ribose) polymerase and Ku autoantigen form a complex and synergistically bind to matrix attachment sequences. J Biol Chem 1999;274:20521–8.

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