Silencing Mediator for Retinoid and Thyroid Hormone Receptor and Nuclear Receptor Corepressor Attenuate Transcriptional Activation by the β-Catenin-TCF4 Complex*

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β-Catenin is a multifunctional mediator of cellular signaling and an oncogene. Nuclear β-catenin, when complexed with members of the T-cell factor (TCF)/leukocyte enhancer factor family of DNA-binding proteins, mediates transcriptional activation important for embryonic development and adult cell homeostasis. Deregelation of intracellular levels of β-catenin is an early event in the development of a variety of cancers. We observed that the proteins silencing mediator for retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor (NCoR) are negative regulators of transcription induced by the β-catenin-TCF4 complex. Overexpression of SMRT and NCoR attenuated the transcription of β-catenin-TCF4-specific reporter gene and of CCND1, an endogenous β-catenin target gene. Knockdown of endogenous SMRT or NCoR by short interfering RNA augmented the β-catenin-TCF4-mediated reporter gene expression. Glutathione S-transferase pulldown experiments showed there was a direct physical association of SMRT and NCoR with both β-catenin and TCF4.

DNA-protein interaction studies revealed that the interactions between either SMRT or NCoR and β-catenin or TCF4 occurred at the promoter regions of CCND1 and other target genes. These findings demonstrate an important role for corepressors SMRT and NCoR in the regulation of β-catenin-TCF4-mediated gene transcription.

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β-Catenin is an important nuclear effector of the Wnt signaling pathway that plays a critical role in cell fate determination, tissue homeostasis, and tumorigenesis (1). β-Catenin is sequestered in the cytoplasm and functions at both the cell membrane and the nucleus. At the cell membrane β-catenin binds to a complex that regulates E-cadherin-mediated epithelial cell adhesion (2). In the nucleus β-catenin binds to members of the T-cell factor (TCF)/leukocyte enhancer factor (LEF) family of proteins to form a heterodimeric transcription complex that activates gene expression downstream from WNT signals (1). In the absence of WNT signals, the cytosolic pool of β-catenin is bound to axin and to the adenosomatous polyposis coli protein that favor phosphorylation of β-catenin at N-terminal Ser/Thr residues by casein kinase Iα and glycogen synthase kinase 3β leading to ubiquitination and degradation by the 26S proteasome (3).

When nuclear β-catenin is of low abundance, TCF/LEF proteins that have DNA binding domains, but lack transcriptional activation function, act as transcriptional repressors, recruiting Groucho/transducin-like enhancer proteins, which in turn recruit histone deacetylase (HDAC) to repress the target gene promoters (4, 5). Changes in chromatin structure are required to relieve the transcriptional repression mediated by these protein complexes. In response to WNT signals, β-catenin phosphorylation is attenuated, leading to increased β-catenin levels in the cytoplasm and the nucleus, where β-catenin binds to TCF/LEF, displaces the corepressor complexes containing HDACs, and enhances transcriptional activation of WNT-responsive target genes (6–8). Transcriptional activation by β-catenin-TCF4/LEF is mediated by recruitment of basal transcription machinery (9) and is enhanced by interaction with many other coactivators, such as the histone acetylase CBP/p300 (10, 11), the p160 coactivator GRIP1 (12), a component of the SWI/SNF chromatin-remodeling complex, Brg-1 (13), and the Legless-Pygopus complex (14).

Silencing mediator for retinoid and thyroid hormone receptor (SMRT) and the closely related protein nuclear receptor corepressor (NCoR) were initially identified as nuclear receptor corepressors (15–17). Both SMRT and NCoR are essential for transcriptional repression by unliganded nuclear receptors, such as the thyroid and retinoic acid receptors (18). In addition, corepressors SMRT and NCoR also interact with antagonist-bound steroid receptors, including androgen receptor (19–21), estrogen receptor (22, 23), glucocorticoid receptor (24, 25), and progesterone receptor (24–26), and may play an important role in the regulation of steroid receptor action. Both SMRT and NCoR interact directly with multiple HDACs, including HDAC3, HDAC4, HDAC5, and HDAC7 (27–30), and may associate with HDAC1 and HDAC2 via the Sin3 protein (31, 32). HDAC3 is found in a tight complex with SMRT and NCoR,
and its enzymatic activity completely depends on association with the deacetylase activation domain of either SMRT or NCoR (27, 30, 33). The recruitment of HDACs enables both SMRT and NCoR to modulate the chromatin structure, thus silencing transcription.

Even though SMRT and NCoR were initially linked with transcriptional repression by nuclear receptors, there is evidence that they also play roles in transcriptional repression by other transcription factors, such as the leukemia chimeric fusion protein PLZF (34, 35), Notch-binding protein CBF-1 (36), members of the AP (activator protein)-1 family (37–39), NF-κB factors (38–40), homeodomain-containing proteins (41–43), Eto (44, 45), and the E-26-transforming specific domain proteins Tel (46, 47) and MEF2c (16). The many associations of SMRT and NCoR suggest that the corepressors may play diverse roles in mediating active transcriptional repression during development. Indeed, deletion of the murine NCoR gene resulted in embryonic lethality and severe developmental defects in the lymphocytic and erythropoietic lineages and in the central nervous system (48).

Here we show that the SMRT and NCoR directly interact with β-catenin and TCF4, both in vitro and in vivo, and that overexpression of either SMRT or NCoR attenuates the transcriptional activity of β-catenin-TF4 target genes, such as CCND1 by a mechanism dependent on the TCF4-binding element (TBE). Knockdown of either endogenous SMRT or NCoR expression augments the transactivation of β-catenin-TF4 reporter genes. Thus, our results suggest that both SMRT and NCoR function as corepressors of β-catenin-TF4-mediated signaling pathway by negatively regulating the expression of WNT target genes.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The Renilla null luciferase reporter was purchased from Promega Corp. (Madison, WI), and pFR-LUC was from Stratagene (La Jolla, CA). TCF4 plasmid and OT-Luc reporter vectors were provided by Kenneth Kinzler (The Johns Hopkins Oncology Center, Baltimore, MD). β-Catenin, GAL4/β-catenin, GAL4/β-catenin-ARM, GAL4/β-cateninΔN, and GAL4/β-cateninΔC have been described previously (49, 50). The cDNA clones encoding NCoR and s-SMRT were gifts from Drs. Mitch Lazar (University of Pennsylvania School of Medicine, Philadelphia) and Dr. Ronald Evans of Salk Institute, La Jolla, CA, respectively. VP16/NCoR.ID (amino acids 1944–2453), VP16/s-SMRT.ID (amino acids 982–1495), and GST/NCoR.ID and GST/s-SMRT.ID (amino acids 982–1495), and GST/NCoR.ID and GST/s-SMRT.ID were kindly provided by Dr. Mitch Lazar (University of Pennsylvania School of Medicine, Philadelphia). GST-β-catenin mutant constructs were supplied by Dr. Andreas Hecht (Institute for Molecular Medicine and Cell Science, University of Freiburg, Freiburg, Germany). pcDNA3-HA-TCF4 mutant constructs were provided by Dr. Jaw-Jou Kang (Institute of Toxicology, College of Medicine, National Taiwan University). The −1745 human CCND1 promoter reporter construct containing 1745 bp upstream of the transcription initiation site, CCND1-Luc, was provided by Dr. Chenguang Wang (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia). pSilencerH1 3.0 vector (Ambion) was used to make SMRT and NCoR small interfering RNA (siRNA) constructs. The target sequences were 5′-AAGAAGGGATCCGATTCGGGA-3′ for NCoR and 5′-AAGCTGAAGGAGGCAGCAAA-3′ for SMRT.

**Cell Culture and Transfection**—Monolayer cultures of CV-1, 293T, and SW480 cells were grown as described previously (49, 50). For transient transactivation assays, cells were plated onto 24-well plates at a density of 4 × 10^4/well, and cells were transfected for 16 h using Lipofectamine (Invitrogen) as recommended by the supplier. For each well of a 24-well plate, we used 100 ng of reporter (OT-Luc, FR-LUC or CCND1-Luc) and 10 ng of Renilla plus various combinations of other expression vectors. Equimolar amounts of expression vectors (pCMX) lacking NCoR or SMRT were included to keep the molarity of each vector constant, with the total transfected DNA brought to 300 ng/well with pBSK+ unless otherwise indicated. After transfection, the cells were cultured for an additional 24 h in complete medium and harvested in 1X Passive Lysis Buffer (100 μl/well; Promega). Cell lysates (30 μl) were used to assay for luciferase activity using the Dual-Luciferase® assay system from Promega according to the supplier. The data were then normalized for the cotransfected Renilla activity.

**GST-Pulldown Assays**—GST-pulldown assays were performed as described previously (49, 50). Briefly, GST fusion proteins (GST-β-catenin, GST-SMRT, and GST-NCoR) were expressed in Escherichia coli and isolated as described before. ^35S-Labeled β-catenin, VP16/SMRT.ID, VP16/NCoR.ID, and TCF4 mutants were produced in vitro using Tnt-coupled/recombinant bacteriophage reticulocyte lysate system (Promega) according to the manufacturer’s recommendations in the presence of ^35S)methionine (Amersham Biosciences). Sonicated bacterial lysates containing overexpressed GST or GST fusion proteins were linked to glutathione-Sepharose beads. Immobilized GST or GST fusion proteins were then incubated overnight at 4 °C with either ^35S-labeled proteins or cell lysates. After extensive washing, the immobilized proteins were removed from the beads by heating at 90 °C for 5 min in 40 μl of 2X SDS loading buffer. The proteins were then separated on 4–20% SDS-polyacrylamide gels, and the bound proteins were visualized by autoradiography for ^35S-labeled proteins or Western blotting for cell lysates.

**RNA Extraction and RT-PCR**—Total RNA was isolated using the RNeasy mini kit (Qiagen, Chatsworth, CA), according to the manufacturer’s recommendations. Semiquantitative one-step RT-PCR was performed according to the manufacturer’s recommendations (Qiagen). We routinely performed the PCR for at least four different cycles to make sure that the reaction was in the linear range. PCR products were separated on agarose gels, and the densities of bands were analyzed. The primers used in the RT-PCR were as follows: CCND1, forward 5′-CCGAGAAGCTGCTGATCCTAC-3′ and reverse 5′-CGCCATCTGGCATTGGTA-3′; β-actin, forward 5′-GGCAGGCGGTGGTACTCT-3′ and reverse 5′-GTGGCCGCTA-3′; NCoR, forward 5′-GCTGATGAGGGTGCAG-3′ and reverse 5′-ATGTTGAGGATGGTTGAG-3′; and reverse 5′-GCTGATGAGGGTGCAG-3′.
Western Blotting—Cell extracts were prepared, and Western blotting analysis was performed as described previously (49, 50) using the following antibodies: anti-β-catenin (BD Transduction Laboratories), anti-TCF4 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-CCND1 (NeoMarker, Freeman, CA), anti-FLAG M2 (Sigma), anti-SMRT (BD Transduction Laboratories), anti-GAL4(DBD) (RK5C1) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-VP16 (14-5) (Santa Cruz Biotechnology), anti-histone H1 (AE-4) (Santa Cruz Biotechnology), and anti-β-actin (Sigma).

Chromatin Immunoprecipitation (ChIP) Assay and Re-ChIP—ChIP assay was performed as described (51, 52) with modifications. In brief, the cells (SW480 or 293T) were fixed by adding formaldehyde to the medium to a final concentration of 1%. After protein-DNA cross-linking for 10 min at room temperature, glycine was added to a final concentration of 125 mM, and the cells were rinsed with phosphate-buffered saline (three times), harvested into lysis buffer (50 mM Hepes-KOH, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 1X complete mini protease inhibitor mixture (Roche Applied Science), and incubated for 10 min at 4 °C. Cell lysates were centrifuged, resuspended in wash buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1X complete mini protease inhibitor mixture), and incubated for 10 min at 4 °C.

Resulting nuclei were centrifuged and resuspended in RIPA buffer (10 mM Tris-HCl, pH 8.0, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM EDTA, 0.5 mM EGTA, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, and 1X complete mini protease inhibitor mixture). Chromatin was sonicated to an average DNA length of 500–1000 bp using Misonix Sonicator 3000 with a microtip (eight times for 10 s at setting 4). Sonicated samples were centrifuged, precleared by incubation with normal IgG and protein G-agarose/salmon sperm DNA (Sonicated samples were centrifuged, precleared by incubation with normal IgG and protein G-agarose/salmon sperm DNA), and antibody-bound chromatin fragments were eluted from the beads with 1% SDS in 0.1M NaHCO3. Cross-links were reversed by heating at 65 °C overnight, and DNA was purified using QIAquick PCR purification system (Qiagen) and analyzed for CCND1 and GAPDH gene sequences by using PCR. PCR was performed using Platinum PCR SuperMix (Invitrogen). Control reactions with genomic DNA were always carried out alongside the immunoprecipitated samples. PCRs, 25:1, were programmed for 22–35 cycles with 0.5–1 µl of DNA samples. Each cycle consisted of a 45-s denaturation at 95 °C, a 45-s annealing at 55 °C, and a 45-s elongation at 72 °C. Promoter-specific primer sets used to amplify the DNA fragments were as follows: CCND1 promoter, forward 5’-CGGACTACAGGGAGTTTGTGTTG-3’ and reverse 5’-TCCAGCATCCAGTGCGACGAT-3’; DKK1 promoter, forward 5’-CACTATTGCACCCACTGAG-3’ and reverse 5’-CAGACCGTGAGATTCAAG-3’; AXIN2 promoter, forward 5’-CTGGAGCCCGGCTGCGCTTTGATAA-3’ and reverse 5’-CGGCCTCCGAA-TCCATCGCTCTGA-3’; GAPDH promoter, forward 5’-AAAAGCGGGGAGAAATAGG-3’ and reverse 5’-CTAGCCTCCGGGGTTTCTCTC-3’.

In Re-ChIP experiments, sonicated and precleared samples were first immunoprecipitated with anti-TCF4 antibody as described above. The antibody-bound chromatin fragments were then eluted by incubation for 30 min at 37 °C in 50 µl of 10 mM dithiothreitol. After centrifugation, the supernatant was diluted 20 times with RIPA buffer and subjected again to the ChIP procedure with either normal IgG or antibodies against β-catenin, SMRT, or NCoR.

Cell Proliferation Assay—SW480 cells were plated onto 48-well plates (1.5 × 10⁴ per well) and transfected with 150 ng/well of control vector pSilencerH1 3.0 or pSilencer-NCoR, pSilencer-SMRT, or both. At 16 h after transfection, fresh complete medium containing 10% fetal bovine serum was added, and cells were cultured for 5 days. Cells were visualized by crystal violet staining and quantified by measurement of absorbance at A₅₇₀.

Statistical Analysis—Unless otherwise noted, values shown represent mean ± S.D. The differences between groups were analyzed for statistical significance by the two-tailed Student’s t test using the program GraphPad Prism software version 4.02 (GraphPad Software, San Diego, CA). p < 0.05 was considered significant. In figures the following symbols are used to represent p values: *, p < 0.05; **, p < 0.01; ***, p < 0.001; and ****, p < 0.0001.

RESULTS

SMRT and NCoR Affect TCF4-mediated Reporter Gene Expression—To study the effect of corepressors SMRT and NCoR on TCF4-mediated transcription, we first analyzed the effect s-SMRT and NCoR have on TCF4-dependent reporter activity in CV-1 cells. S-SMRT (originally called SMRT or TRAC-2, and in our paper we still use SMRT instead of s-SMRT) is the truncated form of the full-length SMRT (called SMRTα or SMRTe). S-SMRT lacks most of the N-terminal repression/cofactor docking sites found in SMRT (53) (Fig. 1). CV-1 cells were cotransfected with the OT-Luc reporter together with β-catenin and increasing amounts of either NCoR or SMRT (0–200 ng/well). Both SMRT and NCoR significantly inhibited TCF4-mediated transactivation of the reporter in a dose-dependent manner (Fig. 2A). SMRT appeared to be more potent because maximal inhibition was achieved with 50 ng/well of the SMRT expression plasmid compared with 100 ng/well of the NCoR expression plasmid. The lesser inhibitory effect of NCoR on β-catenin/TCF4-mediated
transactivation as compared with SMRT might have been due to a lower expression level of NCoR as compared with SMRT (Fig. 2B). A fixed amount of SMRT expression plasmid inhibited reporter expression driven by increasing amounts of β-catenin (Fig. 2C). SMRT also inhibited TCF4-mediated transcription from the −1745 CCND1 promoter, showing that the effect was seen with more than one reporter construct (Fig. 2D). In contrast NCoR expression had no effect on the CCND1-driven reporter construct. Again, this might have been due to the low expression level of transfected NCoR in this system.

Physical Interaction of SMRT and NCoR with β-Catenin—To evaluate whether there was a direct physical interaction between either SMRT or NCoR and β-catenin, we performed a modified mammalian two-hybrid assay. CV-1 cells were transfected with Gal4/β-catenin (Gal4/βcat) constructs that contained full-length or truncated β-catenin, corepressors SMRT, NCoR (Fig. 1), or the corresponding empty vector as a control, and the reporter construct pFR-Luc. The Gal4 segment of the fusion protein binds to the FR-Luc reporter, and the β-catenin fragment initiates assembly of the transcriptional complex. The full-length and truncated Gal4/β-catenin constructs all were active at initiating transcription, and each was inhibited by either SMRT or NCoR (Fig. 3A). Because each of the Gal4/β-catenin constructs contained the central β-catenin armadillo repeat region, which is important for interaction of β-catenin with TCF4, the data suggested that the armadillo repeat region may also be the domain for the association of β-catenin with either SMRT or NCoR. In this experiment we again observed that the apparent effect of SMRT was greater than that of NCoR because of the lower expression level of NCoR as compared with that of SMRT.

We performed a modified mammalian two-hybrid experiment with VP16/SMRT.ID or VP16/NCoR.ID. VP16/SMRT.ID and VP16/NCoR.ID are the C-terminal regions of corepressors that contain the interaction domains (ID) responsible for the association with nuclear receptors (54, 55). Gal4/βcatFL, Gal4/βcatΔN, and Gal4/βcatΔC

FIGURE 1. Construct maps. Schematic representations of the Gal4/β-catenin (A) and corepressors SMRT/NCoR (B) used in this study. Gal4-DBD fusion proteins of wild-type and deletion mutants of β-catenin were used to map the domain required for association with SMRT and NCoR, and full-length NCoR, s-SMRT, and VP16 fusion proteins VP16/NCoR.ID and VP16/SMRT.ID were used in mammalian two-hybrid assays.

FIGURE 2. SMRT and NCoR repress gene transcriptional activity of TCF4. A, triplicate wells of CV-1 cells were transiently transfected with the OT-Luc (100 ng/well), TCF4 (10 ng/well), β-catenin (10 ng/well), increasing amounts of either SMRT or NCoR (0, 50, 100, and 200 ng/well), the molar equivalent of empty vector (pCMX), and 10 ng/well null Renilla for normalization. B, cells were transfected with increasing amounts of SMRT/NCoR as described in A, and Western blot analysis of the expression of NCoR and SMRT was performed with appropriate antibodies as described under “Experimental Procedures.” C, CV-1 cells were transiently transfected as described above except that a constant amount of SMRT (25 ng/well) and increasing amounts of β-catenin (0, 10, 50, and 100 ng/well) were used. D, CV-1 cells were transiently transfected with CCND1 promoter reporter (CCND1-Luc, 100 ng/well), increasing amounts of SMRT or NCoR (0, 25, 50, and 100 ng/well), plus the molar equivalent of pCMX. After transfection, cells were cultured in fresh medium for 48 h, harvested, analyzed, and plotted as described under “Experimental Procedures.” Data represent mean values of triplicate experiments, and standard deviations are shown by error bars. A, C, and D, p values are shown for comparison versus empty vector controls. RLU, relative luciferase units.
values are shown for comparisons of the VP16/SMRT.ID fusion protein (Fig. 3) even though the VP16/SMRT.ID induced a higher level of activation by both VP16/SMRT.ID and VP16/NCoR.ID (Fig. 3). The interactions of either SMRT or NCoR and β-catenin were further examined by GST-pulldown assay using GST–β-catenin constructs and in vitro translated SMRT or NCoR constructs. Either VP16/SMRT.ID or VP16/NCoR.ID was pulled down by GST–β-catenin but not by GST alone (Fig. 4A), confirming the results of the modified mammalian two-hybrid assay in Fig. 3D. Equal amounts of VP16/NCoR.ID and VP16/SMRT.ID gave rise to equal binding in this assay. The GST-pulldown assay indicates that the physical associations of NCoR and SMRT with β-catenin are comparable and are consistent with our interpretation that differences in the β-catenin/TCF4-mediated transactivation assay were due primarily to the lower expression of the NCoR vector as compared with SMRT. We also performed a GST-pulldown assay with GST-NCoR.ID and β-catenin from cell lysates. GST-NCoR.ID is a fusion protein between GST and the C terminus of NCoR (residues 1944–2453). With this fusion protein, we were able to demonstrate the binding of NCoR to β-catenin from either LNCaP or 293T cells. The binding of β-catenin from LNCaP cells to GST-NCoR.ID was stronger than that from 293T cells reflecting the disparity in input levels of protein (Fig. 4B).

To determine the regions of β-catenin required for the association with corepressor IDs, GST fusion constructs of different β-catenin fragments (Fig. 4C) were used. GST–β-catenin-(120–683) bound strongly to VP16/SMRT.ID and VP16/NCoR.ID compared with the fusion construct with full-length β-catenin (Fig. 4D). The autoradiography exposure time for Fig. 4D was much shorter than that for Fig. 4A, accounting for the apparent differences in full-length β-catenin binding between the two panels. Except for GST–β-catenin-(120–683) and full-length β-catenin, all other GST–β-catenin fusion constructs did not bind to either SMRT or NCoR. GST–β-catenin-(120–683) contained the armadillo repeat units plus about 20 amino acids additionally at both the N and C termini. These data suggest that armadillo repeat regions are required for the association between β-catenin and the corepressors IDs.

Physical Interaction of SMRT and NCoR with TCF4—SMRT and NCoR may inhibit the β-catenin/TCF4-mediated transactivation by direct association with either β-catenin, with TCF4, or with both. To determine whether SMRT and NCoR might also be able to interact directly with TCF4, we performed
Endogenous SMRT and NCoR Are Recruited to the TCF4-binding Sites in Promoters of Endogenous Target Genes—

CCND1 is one of the well documented transcription targets of β-catenin. To obtain direct evidence that endogenous corepressors SMRT and NCoR were recruited to TCF4-binding elements (TBE) in the CCND1 promoter, chromatin immunoprecipitation (ChIP) analysis was performed. The cross-linked and sheared nuclear fractions from SW480 cells were subjected to immunoprecipitation with antibodies directed against SMRT, NCoR, TCF4, and β-catenin, and the precipitated DNA was analyzed by PCR amplification using a primer set specific for DNA fragment encompassing the TBE sites of the CCND1 promoter. PCR amplification using the primer set produces a 402-bp product from the CCND1 promoter with the sheared genomic DNA as a template (Fig. 6A, input). TCF4 and β-catenin were preferentially recruited to the TBE sites in the CCND1 promoter. Similarly, corepressors SMRT and NCoR were also preferentially recruited to the same region in CCND1 promoter. Both mouse and goat IgG controls showed equally low background levels. To extend our findings, a different set of ChIP assays was performed with SW480 cells, and it was found that SMRT and NCoR could also be recruited to the TBE sites in the AXIN2 gene promoter, another well documented target gene of Wnt/β-catenin signaling (Fig. 6A). To confirm that SMRT and NCoR were not binding to the genomic sequences in a nonspecific manner, we did ChIP assays with PCR primers specific for the upstream region of the GAPDH promoter sequence that does not contain any known TBE sites. There
noprecipitates were eluted by incubation with 10 mM dithiothreitol at 37 °C for 30 min, diluted 20 times with TBE sites of the GAPDH promoter region of GAPDH, which does not contain any known TBE. PCR products were resolved by agarose gel and stained with ethidium bromide (Fig. 6A), indicating that corepressors SMRT and NCoR specifically bound to the TBE sites of all tested target genes tested (Fig. 6A). Not surprisingly, TCF4 could be constitutively recruited to the TBE sites of target genes when SMRT and NCoR were not released from the TBE sites upon β-catenin binding, again suggesting that the binding of TCF4, β-catenin, and corepressors SMRT and NCoR were not mutually exclusive.

**Overexpression of SMRT and NCoR Inhibited the Expression of Endogenous CCND1—** We next wanted to demonstrate the effects of SMRT and NCoR on CCND1 expression in SW480 cells that have constitutively activated β-catenin. SW480 cells were transfected with increasing amounts of either SMRT or NCoR, and CCND1 mRNA and protein levels were determined by semi-quantitative RT-PCR and Western blot analysis. As we observed in CV-1 cells, the expression level of transfected SMRT in SW480 cells was again much higher than that of nuclear β-catenin, treatment of cells with LiCl caused a progressive increase in β-catenin binding to the TBE sites in the promoter regions of all target genes tested (CCND1, c-MYC, DKK1, and AXIN2, Fig. 7, B–E). Not surprisingly, TCF4 could be constitutively recruited to the TBE sites. Interestingly, the recruitment of SMRT and NCoR to the TBE sites of all tested target genes was not influenced by the treatment with LiCl. This result indicated that the recruitment of SMRT and NCoR to the TBE sites may not be due primarily to β-catenin. Fig. 7 also indicated that coexpressors SMRT and NCoR were not released from the TBE sites upon β-catenin binding, again suggesting that the binding of TCF4, β-catenin, and corepressors SMRT and NCoR was not mutually exclusive.

**FIGURE 6. Recruitment of SMRT and NCoR to the endogenous TCF4-binding sites in promoters of target genes.** A, ChIP analysis was performed with chromatin isolated from SW480 cells, and PCRs were performed using specific primers covering the TBE sites of the CCND1 and AXIN2 promoters or primers amplifying the promoter region of GAPDH, which does not contain any known TBE. PCR products were resolved by agarose gel and stained with ethidium bromide (left panel), and the percentage of the signal corresponding to each PCR product was determined by densitometric scanning as indicated on the right panel that is representative of three different experiments. B and C, ChIP Re-ChIP assays. Chromatin prepared from SW480 cells was first immunoprecipitated (1st IP) with TCF4 antibody (B) or β-catenin antibody (C). After extensive washing, immunoprecipitates were eluted by incubation with 10 mM dithiothreitol at 37 °C for 30 min, diluted 20 times with RIPA buffer, followed by a second immunoprecipitation (ChIP Re-IP) with the indicated antibodies as described under "Experimental Procedures." Specific primers sets covering the TBE sites of the CCND1, AXIN2, and DKK1 promoters were used for PCR.
The expression level of endogenous CCND1 mRNA in SW480 cells was markedly decreased by both SMRT and NCoR, whereas the levels of \( \beta \)-H9252-actin mRNA were unaffected (Fig. 8B). Western blot analyses showed that expression of either SMRT or NCoR could significantly inhibit the expression of cyclin D1. To ask whether expression of exogenous SMRT and NCoR had any significant effect on the expression of \( \beta \)-catenin and TCF4, the same membranes were exposed to antibodies against \( \beta \)-catenin and TCF4. Expression of either exogenous SMRT or NCoR had no significant effect on the expression of endogenous \( \beta \)-H9252-actin and TCF4 (Fig. 8C). These data suggested that the corepressors SMRT and NCoR modulated the \( \beta \)-H9252-actin/TCF4-mediated transcription because of direct interaction with TCF4 and \( \beta \)-catenin rather than via alterations in the expression levels of TCF4 or \( \beta \)-catenin.

Knockdown of Endogenous SMRT and NCoR Increased TCF4 Transcriptional Activity and Promoted Cell Growth—To address further the potential effect of endogenous corepressors SMRT and NCoR on TCF4 transcriptional activity, we knocked
down the expression of endogenous SMRT and NCoR with siRNA in 293T cells, and the β-catenin/TCF4-mediated transcriptional activities were monitored by transactivation assays. The SMRT and NCoR siRNA constructs used in this experiments could knock down the expression of endogenous corepressors SMRT and NCoR (17) (Fig. 9A). In the transactivation assay, the reporter activity was very low if β-catenin was not overexpressed or activated (Fig. 9B, control). Therefore, we used LiCl, a commonly used inhibitor of glycogen synthase kinase-3β (56), to abrogate β-catenin phosphorylation. When cells were treated with 20 mM LiCl overnight, the OT-Luc reporter activity was induced dramatically (Fig. 9B, LiCl). In the presence of LiCl, the TCF4-mediated reporter activity was further augmented by knockdown of either SMRT or NCoR. A similar experiment was performed with the CCND1 promoter-driven reporter (Fig. 9C). Knockdown of either endogenous SMRT or NCoR increased the CCND1 promoter activity in the absence or presence of LiCl. Taken together, these results provided further evidence that endogenous corepressors SMRT and NCoR play a role in the regulation of β-catenin signaling.

Previous studies have shown that down-regulation of β-catenin by either siRNA or antisense oligonucleotides could inhibit SW480 cell growth (57, 58). We next tested the effect of corepressor knockdown by siRNA on cell growth of SW480 cells. As shown in Fig. 9, D and E, knockdown of SMRT and NCoR individually could induce a slight but statistically significant increase in cell growth. Knockdown of SMRT and NCoR simultaneously led to a further increase in cell growth, suggesting that there is a functional redundancy of SMRT and NCoR.

**DISCUSSION**

Increasing evidence suggests that β-catenin signaling is able to undergo cross-talk with and integrate multiple intracellular signals to coordinate cellular functions, such as those involving forkhead box O transcription factors (59), transforming growth factor β (60), RAS signaling (61), and AP-1 signalings (62). More recently, we and others have found that β-catenin signaling can undergo cross-talk with androgen receptor signaling (21, 49, 50, 63, 64). Androgen receptor is a member of steroid/nuclear receptor superfamily (65). It has been well documented that the mechanism of transcriptional activation by androgen receptor involves many associated proteins, including coactivators and corepressors (18). Interestingly, some of the coactivators, such as CBP and p300, also play pivotal roles in the β-catenin/TCF4-mediated transactivation (10, 11), and the p160 family coactivator GRIP1 has been reported to enhance β-catenin/TCF4-mediated transactivation synergistically with β-catenin (12).
Our experiments show that SMRT and NCoR, two important corepressors for androgen receptor and other steroid/nuclear receptor-mediated transcriptional repression, play a role in modulation of β-catenin signaling. The β-catenin/TCF4-mediated transcription of two different reporter constructs and of endogenous CCND1 was down-regulated by expression of exogenous SMRT or NCoR. Importantly, knockdown of endogenous SMRT or NCoR enhanced the activities of β-catenin/TCF4-responsive reporters and promoted cell proliferation. These results indicated that corepressors SMRT and NCoR repressed the β-catenin/TCF4-mediated transactivation, and this repression occurred in the presence of native levels of endogenous corepressors. Interestingly, the attenuated β-catenin/TCF4-mediated transactivation and the decreased expression of CCND1 are not because of the decreased expression of β-catenin or TCF4. Thus the effect of SMRT and NCoR may occur at transcriptional levels by either directly inhibiting the binding of β-catenin, TCF4, and the TCF4 binding region of target genes or by inducing chromatin modification. The latter explanation is supported by the fact that corepressors SMRT and NCoR are essential for transcriptional repression by unliganded nuclear receptors and antagonist-bound steroid receptors by directly associating with various HDACs, thereby modulating the chromatin structure and silencing transcription (66). In addition, our results show that corepressors SMRT and NCoR directly interact with both TCF4 and β-catenin. The mammalian two-hybrid experiments and GST-pulldown assays indicate that the C-terminal nuclear receptor interaction domains are required for the association of corepressors with both β-catenin and TCF4. For β-catenin and TCF4, the armadillo repeat domains and high mobility group domains, respectively, are required for the interaction with SMRT and NCoR. These results demonstrate that the corepressors SMRT and NCoR repress β-catenin signaling by association with both β-catenin and with TCF4. ChIP analysis showed that endogenous corepressors SMRT and NCoR bind indirectly via β-catenin and the SMRT-HDAC corepressor complex (67). Similar to our studies, it has been demonstrated previously that the homeobox protein heterodimer Hox-pbx, N-CoR, and CBP exist in a single complex and that cAMP-dependent protein kinase-mediated phosphorylation of Hox-pbx by cAMP-dependent protein kinase permits this transcription factor to activate target gene transcription (42, 43).

Because the WNT/β-catenin signaling pathway plays a critical regulatory role in many biological processes, including embryonic development and stem cell maintenance (8), it is not surprising that deregulation of WNT/β-catenin signaling is associated with multiple diseases, including various cancers (2, 68). For example, abnormal activation of the WNT/β-catenin signaling in colorectal cancer is caused by either loss-of-function mutations in APC or gain-of-function mutations in the β-catenin gene, leading to the accumulation of nuclear β-catenin (2, 8, 69, 70). Recent data suggest that corepressors may play an important regulatory role in the development and/or progression of colorectal cancer. In colorectal cancers, IκB kinase α is aberrantly activated and recruited to chromatin concomitantly with the phosphorylation of SMRT and NCoR, leading to an increased affinity of the corepressors for the 14-3-3 adaptor proteins and to the cytoplasmic export of both SMRT and NCoR (71, 72). The findings that aberrant cytoplasmic distribution of SMRT and/or NCoR is seen in colorectal cancer cells lead us to speculate that cytoplasmic redistribution of corepressors SMRT/NCoR further amplifies WNT/β-catenin signaling by diminishing the nuclear pool of SMRT and/or NCoR.

In summary, we have shown that SMRT and NCoR inhibit β-catenin/TCF4-dependent gene expression, including CCND1 (Fig. 10). This is important for understanding the regulation of the Wnt/β-catenin signaling pathway. Because corepressors play critical roles in steroid/nuclear receptor and other transcription factor-mediated transcriptional repression, the data of this study may provide an additional mechanism...
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responsible for the cross-talk between β-catenin/TCF4 and many other transcription factor-mediated signaling pathways.

Acknowledgments—We thank Drs. Kenneth Kinzler, Michael Rosenfeld, Ronald Evans, Mitch Lazar, Andreas Hecht, Jaw-Jou Kang, and Chenguang Wang for providing reagents.

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