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An Antisense Transcript Induced by Wnt/β-Catenin Signaling Decreases E2F4*

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Wnt signaling induces the nuclear accumulation of β-catenin and transcription of specific target genes via the DNA-binding proteins TCF/Lef. Although all known β-catenin target genes encode proteins, genome-wide RNA profiling studies indicate that many transcripts do not have this capability. Transcription factor-binding sites associated with these noncoding transcripts can be identified using unbiased techniques such as serial analysis of chromatin occupancy (SACO). We used this method to identify a β-catenin-regulated antisense RNA expressed in HCT116 colorectal carcinoma cells, a cellular model of activated β-catenin signaling. Genomic signature tags designating putative β-catenin-binding sites mapped to the 3′-untranslated region (3′-UTR) of the *E2F4* gene. We showed that both β-catenin and TCF4 bind to the *E2F4* 3′-UTR site *in vivo*, inducing expression of an *E2F4* antisense transcript. LiCl, which mimics Wnt signaling, also induced expression of the *E2F4* antisense transcript and decreased E2F4 protein levels. This effect was blocked by a cDNA expressing the *E2F4* 3′-UTR sense strand. The antisense-mediated decrease in E2F4 protein was reflected by reduced E2F4 association with specific target genes, including *CCNA2*, *CDC2*, *PCNA*, and *Rad54*. We propose that Wnt/β-catenin signaling may contribute to colorectal carcinogenesis by reducing the level of the E2F4 cell cycle repressor via an antisense mechanism.

The Wnt signaling pathway is central to cell fate decisions critical for development and homeostasis in metazoan organisms (for review, see Refs. 1–5). In the absence of Wnt, cytoplasmic β-catenin resides in a multi-protein complex that includes the adenomatous polyposis coli protein, axin, and glycogen synthase kinase 3β (GSK3β).[2] Within this complex, β-catenin is phosphorylated by GSK3β and targeted for proteasomal degradation. Wnt signaling inactivates GSK3β and stabilizes β-catenin, which allows for its transport into the nucleus. Once in the nucleus, β-catenin associates with members of the TCF/Lef family of DNA-binding proteins and activates gene expression (6, 7). One important consequence of β-catenin signaling is the stimulation of cellular proliferation. Indeed, 90% of sporadic colon carcinomas contain mutations in the canonical Wnt signaling pathway (8, 9). Thus, an understanding of the pathophysiology of colorectal cancers would be greatly enhanced through the identification of novel β-catenin genomic targets.

A frequently cited collection of direct β-catenin targets currently lists only 28 mammalian genes, all of which encode proteins (www.stanford.edu/~rnusse/wntwindow.html). However, recent studies by the FANTOM3 (functional annotation of mouse 3) consortium indicated that the number of transcripts far exceeds the number of protein-coding genes (10). A particularly interesting aspect of the FANTOM3 analysis was the recognition of a vast number of noncoding RNAs, including antisense transcripts (10, 11). These findings are consistent with the observation that transcription factor-binding sites frequently lie outside the typical 5′ promoter regions of protein-coding genes (12–14). Indeed, it has been suggested that some of these atypical regulatory elements could potentially control the expression of noncoding transcripts (12–14). Thus, it seemed reasonable to assume that noncoding as well as coding transcripts might be regulated by the Wnt signaling pathway.

Two experimental techniques have been developed to identify transcription factor-binding sites on a genome-wide level. In ChIP-chip assays, immunoprecipitated chromatin fragments are hybridized to microarrays typically designed to represent 5′-flanking regions (15). Detection of binding sites is limited by the number of features and types of elements in array design. In particular, a 5′ bias in the design of many of the "promoter" type arrays may lead to a severe underestimate of the number of sites regulated by a given transcription factor. More comprehensive tiled arrays enable more extensive coverage, but completely tiled mammalian genome arrays are not yet available. In contrast, sequence-based techniques, such as serial analysis of chromatin occupancy (SACO), identify and localize transcription factor targets in an unbiased manner (13, 16–20). Thus, an advantage of the sequence-based approaches is that they can detect transcription factor-binding sites that might regulate the expression of novel noncoding RNAs.

This report addresses a novel β-catenin-regulated transcript derived from the *E2F4* gene locus. The E2F family of transcription factors contains at least eight members that coordinate expression of genes involved in cell cycle entry, progression,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental methods.

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[2] The abbreviations used are: GSK, glycogen synthase kinase; ChIP, chromatin immunoprecipitation; siRNA, small interfering RNA; SACO, serial analysis of chromatin occupancy; GST, genomic signature tag; UTR, untranslated region.

and exit (for review, see Refs. 21–24). E2F target genes are also involved in DNA repair, cell cycle checkpoints, and apoptosis. E2F1, E2F2, and E2F3a activate genes involved in transiting the G_1/S boundary, whereas E2F3b is a repressor expressed throughout the cell cycle. E2F4 and E2F5 function during G_0 and early G_1 and are also believed to act predominately as repressors. E2F6 and 7 are thought to function as transcriptional repressors as well, but, as is the case for E2F8, their precise roles in cell cycle regulation are not fully understood. Although cell cycle progression is promoted by both β-catenin and the activating E2F family members, these pathways are not believed to intersect. We used SACO to identify a novel β-catenin target that links these two pathways in HCT116 colorectal carcinoma cells, a prototypical model of activated Wnt/β-catenin function. β-Catenin binding to a consensus TCF element within the genomic region representing the *E2F4* 3′-UTR was found to induce expression of an *E2F4* antisense transcript. The antisense transcript in turn reduced E2F4 protein levels and decreased binding of E2F4 to target gene promoters. These observations suggest a novel mechanism for Wnt signaling to activate expression of genes repressed by E2F4.

EXPERIMENTAL PROCEDURES

Cell Culture—HCT116 cells (ATCC number CCL-247) were grown in McCoy's 5A modified medium (ATCC). HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen). H1299 cells were grown in RPMI 1640 medium (Invitrogen). The media were supplemented with 10% fetal bovine serum (Hyclone), 100 units/ml penicillin, 100 units/ml streptomycin, and 5 mM L-glutamine. The cells were maintained at 37 °C and 5% CO_2.

Chromatin Immunoprecipitation—Antibodies used for ChIP included: 3 μg of anti-β-catenin (BD Transduction, 610154), 3 μg of anti-TCF4 (Upstate Biotechnology, 05-511), 3 μg of anti-E2F4 (Santa Cruz, SC1082), 3 μg of anti-β-galactosidase (Promega, Z378B), 3 μg of anti-Gal4 (Santa Cruz, SC577), and 6 μg of rabbit anti-mouse IgG (Jackson ImmunoResearch, 315-005-003). ChIP assays contained 5×10^6 cells and were conducted as reported (25) with the following modifications: chromatin in formaldehyde-fixed cell lysates was sonicated to an average size of ~600 bp using a Misonex cup horn sonicator (5 × 20 s, 140–150 Watt pulses with 60-s rest intervals on ice). The lysates were clarified by centrifugation at 14,000 rpm for 10 min at 4 °C and then incubated with primary antibody overnight at 4 °C. Secondary rabbit anti-mouse IgG was added for 6 h the following day. Immunocomplexes captured with bovine serum albumin/glycogen-blocked protein A-Sepharose (Repligen) were washed, and precipitated DNA fragments were isolated with 10% Chelex-100 (Bio-Rad) as described (25). Isolated DNA fragments were quantified by real time PCR as previously described (13). Primers were designed using MIT Primer3 software (frodo.wi.mit.edu/cgi-bin/primer3_www.cgi) and synthesized at IDT technologies. The sequences are available upon request.

SACO—Briefly, an adapter was ligated to ChIP fragments, and the DNA was amplified using limited PCR. Digestion of the ChIP fragments with NlaIII provides an anchor for ligation of adapters containing a recognition sequence for MmeI. MmeI is a class IIS restriction enzyme that cleaves 20–22 base pairs downstream from its binding site leaving a 5′-NN overhang. The MmeI-digested fragments were self-ligated to form cassette bound "ditags." Each ditag contains two 20–22-base pair genomic signature tags (GSTs), corresponding to two independent β-catenin-binding regions. The cassette-bound ditags were digested with NlaIII and concatamerized, and the resultant fragments were subcloned into the SphI site of pZERO-2 (Invitrogen). A second SphI site in the kanamycin resistance gene in pZERO-2 was mutated using the QuikChange mutagenesis kit (Stratagene) prior to subcloning of the concatamers. For a detailed SACO protocol, see supplemental methods online and Ref. 13.

Plasmids—pME18-Lef, pME18-deltaN67-LEF, and LEF-HDAC were gifts from D. Ayer (Huntsman Cancer Institute, University of Utah). cDNA encoding full-length β-catenin was a gift from M. Wong (Oregon Health and Science University) and was subcloned into pcDNA3.1 (Invitrogen) as a BamHI fragment. Serine 45 was mutated to phenylalanine using pcDNA3.1-β-catenin and primers 5′-ACC ACA GCT CCT TTT CTG AGT GGT-3′ and 5′-ACC ACT CAG AAA AGG AGC TGT GGT-3′ in a site-directed mutagenesis reaction (Stratagene). The pGL3-*E2F4*-3′-Luc construct was made by first PCR amplifying the 3′-UTR of *E2F4* from HCT116 genomic DNA with 5′-GCT AGC TGG AAT GTT AGT AAC TGA GC-3′ and 5′-GGT ACC GCA GAA GCA AGA CAC TGA GGC-3′. The PCR products were subcloned as NheI-KpnI fragments into the pGL3-basic-luciferase vector (Stratagene).

The consensus TCF site 5′-CTTTGAT-3′ was mutated to 5′-CGCTGAT-3′ using pGL3-*E2F4*-3′-UTR and primers 5′-GAA GGT GTC TGT GAC CTC GCT GAT GTG CCT GTT CTC-3′ and 5′-GAG AAC AGG CAC ATC AGC GAG GTC ACA GAC ACC TTC-3′ in a site-directed mutagenesis reaction (Stratagene). The *E2F4* 3′-UTR mammalian expression constructs used pcDNA3.1 (Invitrogen) as the vector backbone. *E2F4* 3′-UTR in the sense orientation was PCR-amplified using 5′-CAT GGA TCC CTG ACA GGG ACA TGC CCT GTG-3′and 5′-CAT GAA TTC GCC GAA ATG AAG AGA GGG TTA TGG-3′. *E2F4* 3′-UTR in the antisense orientation was PCR-amplified using 5′-CAT GAA TTC CTG ACA GGG ACA TGC CCT GTG-3′ and 5′-CAT GGA TCC GCC GAA ATG AAG AGA GGG TTA TGG-3′. Both products were subcloned into pcDNA3.1 as BamHI-EcoRI fragments.

Quantitative Reverse Transcription-PCR—5×10^6 HCT116 cells were lysed using a Qiashredder column (Qiagen). RNA was isolated using the RNeasy kit (Qiagen). Genomic DNA was removed using the DNA free kit (Ambion), and the DNase was removed using a DNase inactivation reagent (Ambion). First strand cDNA was synthesized from 1 μg of DNA-free RNA using a random hexamer primer (Invitrogen) and Superscript III reverse transcriptase (Invitrogen). *α-2-Tubulin* (*tubulin*) was amplified from random primed cDNA with 5′-GGG GCT GGG TAA ATG GCA AA-3′ and 5′-TGG CAC TGG CTC TGG GTT CG-3′ using PCR. The *E2F4*-specific oligonucleotides 5′-GAA TGT TAG TAA CTG AGC TCC C-3′ and 5′-GTC AAC CTC TGA CTG ACA GG-3′ were used to prime first strand cDNA for detection of the *E2F4* antisense tran-

script. The *E2F4* antisense transcript was detected using the *E2F4*-specific primers 5′-CGC AGA GCA GGG GAA CAG GA-3′ and 5′-GCC GCA GAA AGG GAG AAG CA-3′ by PCR. Reactions lacking reverse transcriptase were run in parallel as a control for the presence of genomic DNA. To quantify cDNA, real time PCR was conducted as for ChIP, except 10 ng of cDNA was used as template.

Mapping of the 5′-Capped Nucleotide—A modification of the first choice RNA ligase-mediated rapid amplification of cDNA ends kit (Ambion) was used to identify the 5′-capped nucleotide of the *E2F4* antisense transcript as reported (26).

Luciferase Reporter Assays—For each sample, examined in quadruplicate, 5×10^5 HEK293 cells were seeded in one well of a 12-well plate. The following amounts of plasmid were transfected using ExGen 500 (Fermentas) as indicated in the figure: pGL3-*E2F4* 3′-UTR-Luc or pGL3-*E2F4* 3′-UTR-Luc (mut), 125 ng; pcDNA3.1-β-catenin S45F, 250 ng; pME18F-dnLEF (delta N67) or pME18F-LEF-HDAC, 250 ng; and pCMV-β-Gal, 25 ng. Total DNA transfected was adjusted to 2 μg with pBluescript (Stratagene). Transfection complexes were removed after a 5-h incubation. Luciferase assays were performed as previously described, and the values were normalized to β-galactosidase (27).

Cellular Fractionation—Pelleted HCT116 cells were resuspended in nuclear isolation buffer (10 mM KCl, 10 mM Tris, pH 7.9, 0.625% Nonidet P-40) and allowed to swell on ice for 15 min. After 25 strokes with a Dounce homogenizer, the nuclei were pelleted for 10 min at 4 °C at 3000 rpm (960 × *g*). The soluble fraction was removed to a new tube and centrifuged at 14,000 rpm (20,800 × *g*) to pellet organelles and insoluble material. The soluble fraction is cytoplasm. Total RNA was isolated from nuclei and cytoplasm using TRIzol (Invitrogen). Polyadenylated RNA was purified from total RNA using the Oligotex direct mRNA kit (Qiagen).

Western Blot—Western blots were conducted as previously described (28). Dilutions of antibodies used are: 1:1000 β-catenin (BD Transduction, 610154), 1:200 TCF4 (Upstate Biotechnology, 05-511), 1:10,000 α-tubulin (Sigma, T3526), and 1:1000 E2F4 (Santa Cruz, SC1082).

siRNAs and Transfection of HCT116 Cells—SMARTpool siRNAs directed against β-catenin and E2F4 were from Dharmacon. 100–200 pmol of siRNA were transfected into 5×10^6 HCT116 cells using the Nucleofector Kit V according to the manufacturer's guidelines (Amaxa). siRNAs were incubated with cells for 48 h prior to either ChIP or Western blot analysis. 3 μg of *E2F4* 3′-UTR was transfected into HCT116 cells by nucleofection (Amaxa). Parallel assays using green fluorescent protein indicated that 90–100% of cells were transfected.

Northern Blot—Total RNA from HCT116 and HEK293 cells was isolated using the RNAEasy kit (Qiagen). 10 μg of poly(A)-enriched mRNA was purified using Oligotex resin (Qiagen). Northern blot was performed using the Northern Max kit (Ambion). The *E2F4* 3′-UTR riboprobe was synthesized using the Maxiscript *in vitro* transcription kit (Ambion) with T7 RNA polymerase and linear pcDNA3.1 *E2F4* 3′-UTR sense plasmid. To remove unincorporated [^{32}P]UTP, the probe was purified over NucAway (Ambion) centrifuge columns. The probe (10^7 cpm) was added to 2 ml of ULTRAhybe (Ambion) and hybridized overnight at 68 °C. The blot was rinsed twice with low stringency wash buffer (2× SSC, 0.1% SDS) and twice with high stringency wash buffer (0.2× SSC, 0.1% SDS) for 30 min at 68 °C.

RESULTS

HCT116 cells contain a mutated β-catenin allele that stabilizes the β-catenin protein (9). Stabilized β-catenin is translocated into the nucleus where it activates a genetic program that has only partially been characterized. We used SACO to identify the genomic targets of β-catenin in HCT116 cells, a colon cancer model of activated Wnt/β-catenin signaling.[3] In this approach, transcription factor-binding sites are identified by 20–22-bp genomic tags termed GSTs that are mapped to genomic databases (13). Three tags mapped to the 3′-UTR region of the *E2F4* gene, suggesting that this β-catenin-binding site could demarcate a promoter that regulates an *E2F4* antisense transcript.

We first sought to confirm β-catenin binding to the *E2F4* 3′-UTR sequences using ChIP assays. To test the efficacy of the β-catenin antibody, we assayed its ability to precipitate the promoters of two known β-catenin targets, c-*myc* and *cyclin D1* (29, 30). Enhanced β-catenin binding was seen at both promoters (Fig. 1*A*). To ensure that the binding was specific, we tested several additional sites, including *tubulin* and *GAPDH*, which are not known to be β-catenin target genes, as well as six 5-kilobase genomic regions that lack a consensus TCF site (Fig. 1*A*, labeled *ctrl 1–6*). The signal detected using the β-catenin or β-galactosidase antibodies were equivalent at these nonspecific sites, reflecting background binding. Importantly, significant binding to the *E2F4* 3′-UTR site was detected, confirming that β-catenin binds this region *in vivo* (Fig. 1*A*). β-Catenin is believed to bind DNA primarily via TCF/Lef, and in colon cells, the predominant member of this family is TCF4 (3, 31). To determine whether TCF4 also bound the *E2F4* 3′-UTR sequences, we repeated the ChIP assays using TCF4 antibodies. TCF4 bound the c-*myc* and *cyclin D1* promoters, in agreement with previous studies (Fig. 1*B*) (29, 30, 32). Significant levels of TCF4 binding to the *E2F4* 3′-UTR sequences were also detected, suggesting that β-catenin is recruited to this region via TCF4. As was the case for β-catenin, only background levels of TCF4 binding were detected at *tubulin*, *GAPDH*, or sites lacking TCF consensus motifs. The higher level of binding detected using the TCF4 antibodies probably reflects the fact that β-catenin interacts with DNA indirectly via TCF4.

β-Catenin and TCF4 binding to the *E2F4* 3′-UTR site suggests that this region may regulate expression of a nearby transcript. We used a modified cap trapping assay to identify the 5′ end of the *E2F4* antisense transcript (26). Sequencing of several independent clones established that the 5′ cap localized to a thymidine at position 65790167 on chromosome 16. This region falls between the β-catenin GSTs in the 3′-UTR of *E2F4* (Fig. 2). A consensus TCF4 sequence, 5′-CTTTGAT-3′, was localized in close proximity to the β-catenin GSTs. To test whether this putative 3′-UTR-binding site was functional, we fused a 656-base pair region surrounding the site to a luciferase

[3] G. S. Yochum, S. McWeeney, and R. H. Goodman, manuscript in preparation.

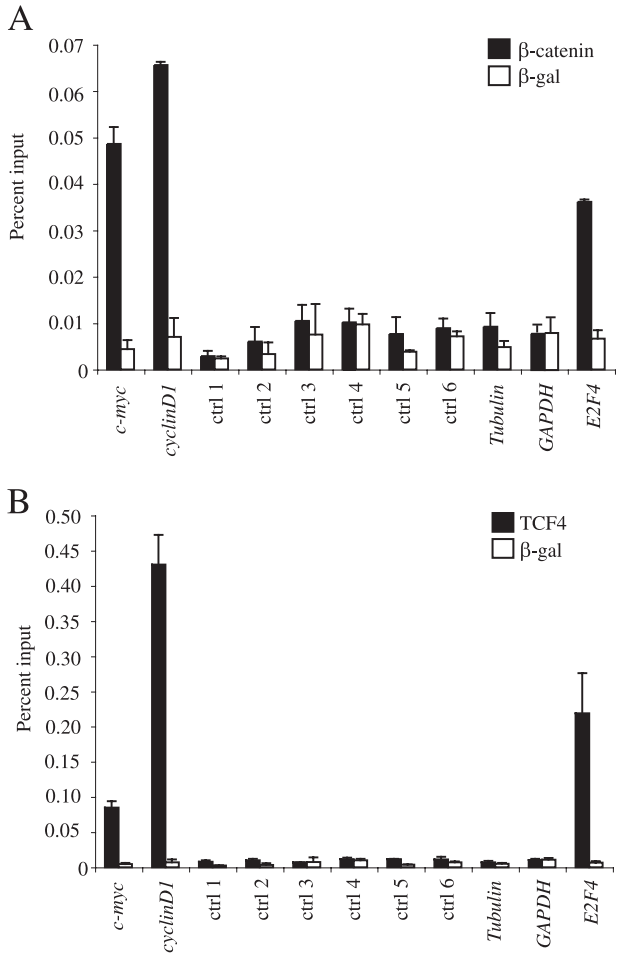

FIGURE 1. **β-Catenin and TCF4 bind the 3′-UTR of *E2F4 in vivo*.** *A*, real time PCR quantitation of DNA fragments precipitated in a ChIP assay using β-catenin antibodies (*dark bars*) or a β-galactosidase (*β-gal*) antibody as a control (*ctrl*, *open bars*). Regions interrogated as negative controls included *tubulin*, *GAPDH*, and six 5-kilobase genomic regions chosen at random that lack a consensus TCF motif. The data are presented as percentages of input. *B*, as in *A* except that anti-TCF4 antibodies were used for immunoprecipitation. The *error bars* represent S.E.

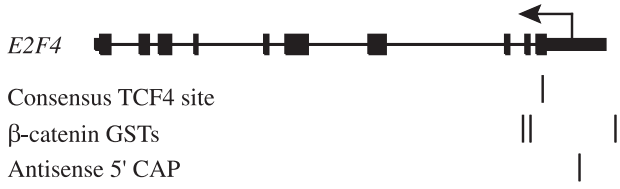

FIGURE 2. **Diagram depicting the positions of β-catenin GSTs, consensus TCF4 site, and the 5′ cap of the antisense transcript relative to the *E2F4* genomic locus.** The *E2F4* gene is depicted with exons as *rectangles*, introns as *horizontal lines*, and the 5′- and 3′-untranslated regions as *thin rectangles*. The locations of the β-catenin GSTs, the consensus TCF site, and the 5′ cap of the antisense transcript are indicated by *vertical lines*. The *arrow* indicates the direction of transcription.

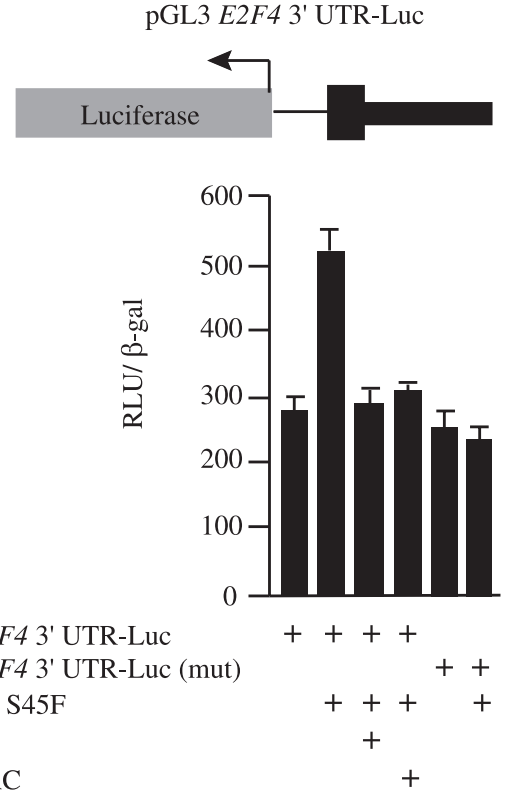

FIGURE 3. **β-Catenin activation of a luciferase reporter driven by a portion of the *E2F4* 3′-UTR is dependent on TCF/Lef.** A 656-base pair portion of the *E2F4* 3′-UTR was subcloned into the pGL3-basic luciferase reporter. This construct, or a construct containing a mutated TCF4 site, along with plasmids encoding the proteins indicated, were transfected into HEK293 cells. 24 h later, luciferase levels were assayed and normalized to β-galactosidase. The *error bars* are S.E. *Top*, diagram of pGL3 *E2F4* 3′-UTR-Luc. *dn*, dominant negative.

reporter. Reporter activity was assayed in HEK293 cells transfected with a β-catenin cDNA containing the same mutation at serine 45 found in many colon carcinomas (5). β-Catenin activated the luciferase reporter and co-transfection of a dominant negative form of Lef, which lacks the β-catenin-binding domain, blocked this activation (Fig. 3). Similarly, co-transfection of a Lef-HDAC fusion protein blocked reporter activation, as did mutation of the consensus TCF4-binding site in the reporter. These results indicate that recruitment of β-catenin to the 3′-UTR site and the subsequent transcriptional induction occur through TCF4. Interestingly, the 5′-capped nucleotide and start site of transcription is upstream from the consensus TCF4 site (Fig. 2). This suggests that β-catenin can regulate promoters upstream of its binding site in a manner similar to c-*myc* (33).

A riboprobe representing a 635-bp portion of the 3′-UTR adjacent to the start site was used in Northern blots to detect the full-length *E2F4* antisense transcript in HCT116 and HEK293 cells. A single band, ~5.5 kb in length, was identified, indicating that the antisense transcript spanned most of the 6.7-kb primary *E2F4* transcript (Fig. 4*A*). Strand-specific real time reverse transcription-PCR demonstrates that the *E2F4* antisense transcript can be detected in polyadenylated RNA isolated from cytoplasmic and nuclear fractions (Fig. 4*B*). To test whether β-catenin signaling regulated expression of the *E2F4* antisense transcript, we treated cells with LiCl that, like Wnt stimulation, inhibits GSKβ (34) and stabilizes β-catenin. Treatment of HCT116 cells with 10 mM LiCl caused a 4-fold increase in the levels of the *E2F4* antisense transcript (Fig. 4*C*). To confirm that the *E2F4* antisense transcript is regulated by the canonical Wnt pathway, we transfected HEK293 cells with plasmids expressing Lef and β-catenin S45F. As has been reported for other β-catenin targets, sig-

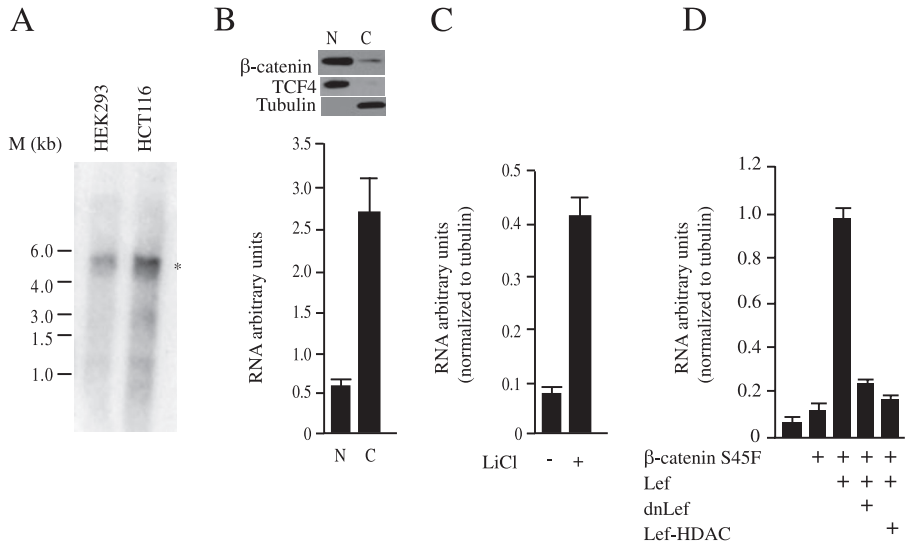

FIGURE 4. **Wnt/β-catenin signaling activates expression of a novel *E2F4* antisense transcript.** *A*, Northern blot of polyadenylated RNA isolated from HEK293 and HCT116 cells. The *E2F4* antisense transcript was detected using a sense *E2F4* 3′-UTR riboprobe. *M*, RNA ladder. *B*, the *E2F4* antisense transcript localizes to the nucleus and cytoplasm. *Top*, nuclear (*N*) and cytoplasmic (*C*) fractions were isolated as described. Western blot analysis using antibodies against β-catenin, Lef, and tubulin demonstrates purity of the fractions. Polyadenylated RNA was isolated, and first strand cDNA was synthesized using an oligonucleotide against the *E2F4* antisense transcript. Quantitative real time PCR was performed using primers designed downstream of the 5′ antisense cap. Serial dilutions of sonicated genomic DNA were analyzed in parallel samples to establish standard curves. *C*, treatment of HCT116 cells with LiCl increases levels of the *E2F4* antisense transcript. The cells were treated with 10 mM LiCl for 48 h, and the *E2F4* antisense transcript was measured from RNA as in *B*. *D*, β-catenin activation of the endogenous *E2F4* antisense transcript requires TCF/Lef. HCT116 cells were transfected with expression plasmids encoding the indicated proteins. 48 h later, RNA was isolated, and quantitative reverse transcription PCR was performed as in *B*. In *C* and *D*, data are normalized to tubulin. The *error bars* are S.E. *dn*, dominant negative.

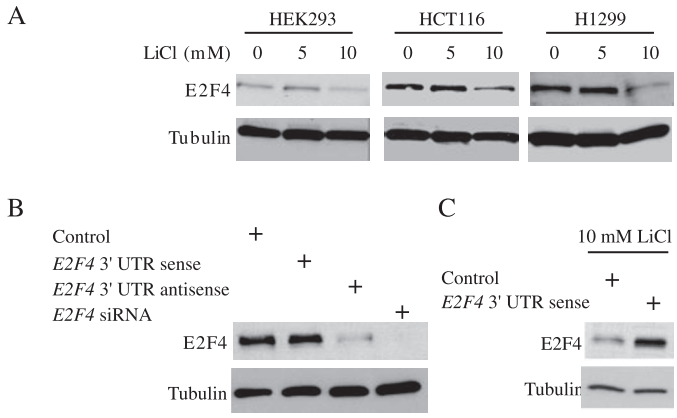

FIGURE 5. **Expression of the antisense transcript decreases E2F4 protein.** *A*, HEK293, HCT116, and H1299 cells were treated with 5 or 10 mM LiCl for 48 h. The protein extracts were subjected to SDS-PAGE and probed with E2F4 or α-tubulin antibodies in a Western blot. *B*, HCT116 cells were transfected with plasmids expressing a portion of the sense or antisense *E2F4* 3′-UTR. 48 h later, the protein extracts were subjected to Western blot as in *A*. The cells were transfected with an siRNA against *E2F4* as a control. *C*, HCT116 cells were transfected with *E2F4* 3′-UTR sense orientation, and 10 mM LiCl was added the following day. Two days later, E2F4 and tubulin proteins were detected by Western blot as in *A* and *B*.

nificant induction of the endogenous *E2F4* antisense transcript by β-catenin required Lef (Fig. 4*D*) (35–38). This activation was decreased upon co-transfection with either dominant negative Lef or a Lef-HDAC fusion protein. Together, these experiments demonstrate that the endogenous *E2F4* antisense transcript is regulated by the canonical Wnt pathway.

If regulation of the *E2F4* antisense transcript is functionally meaningful, its induction should affect the level of E2F4 protein. As shown in Fig. 5*A*, E2F4 protein levels were reduced in three different cell lines treated with LiCl. Similarly, transfecting HCT116 cells with an antisense cDNA corresponding to a 635-base pair portion of the *E2F4* 3′-UTR also caused a significant decrease in E2F4 protein levels (Fig. 5*B*). This decrease was dependent upon the orientation of the cDNA fragment, because expression of the *E2F4* 3′-UTR in the sense orientation had little effect. The antisense-mediated decrease of *E2F4* was not as effective as treatment with *E2F4* siRNAs, however. To link the LiCl effect to expression of the antisense transcript directly, we expressed the sense 3′-UTR fragment in HCT116 cells and, the following day, treated the cells with 10 mM LiCl. Expression of the sense *E2F4* 3′-UTR blocked the LiCl-induced decrease of E2F4 protein (Fig. 5*C*), supporting our hypothesis that the LiCl-induced decrease in E2F4 protein is mediated by the *E2F4* antisense transcript.

To determine whether the *E2F4* antisense regulation was reflected by decreased E2F4 at native promoters *in vivo*, we performed ChIP assays. An E2F4 antibody specifically precipitated several E2F targets in HCT116 cells, including the *CCNA2*, *CDC2*, *PCNA*, and *RAD54* genes (Fig. 6*A*). Control genes *GAPDH* and *tubulin* showed no enrichment when using E2F4 or Gal4 antibodies (Fig. 6*A*). Expression of the antisense, but not the sense, 3′-UTR fragment decreased promoter-bound E2F4 protein at E2F target genes (Fig. 6*B*). LiCl treatment also reduced E2F4 association with target genes (Fig. 6*C*). This reduction was blocked by expression of the sense *E2F4* 3′-UTR, suggesting that the LiCl effect was mediated by the endogenous *E2F4* antisense transcript. Together, these experiments suggest that Wnt/β-catenin signaling can affect levels of E2F4 at critical gene promoters by decreasing E2F4 protein levels.

DISCUSSION

Large scale transcript analyses indicate that most gene activity in mammals does not result in the production of proteins (10). Rather, noncoding RNAs are by far the predominant genomic product (10). The functions of certain noncoding RNAs, including ribosomal RNAs, transfer RNAs, snoRNAs, and others, have been studied for decades. However, other noncoding RNAs, such as microRNAs, have only recently received attention. One surprising finding of large scale RNA profiling was the preponderance of antisense transcripts (11). According

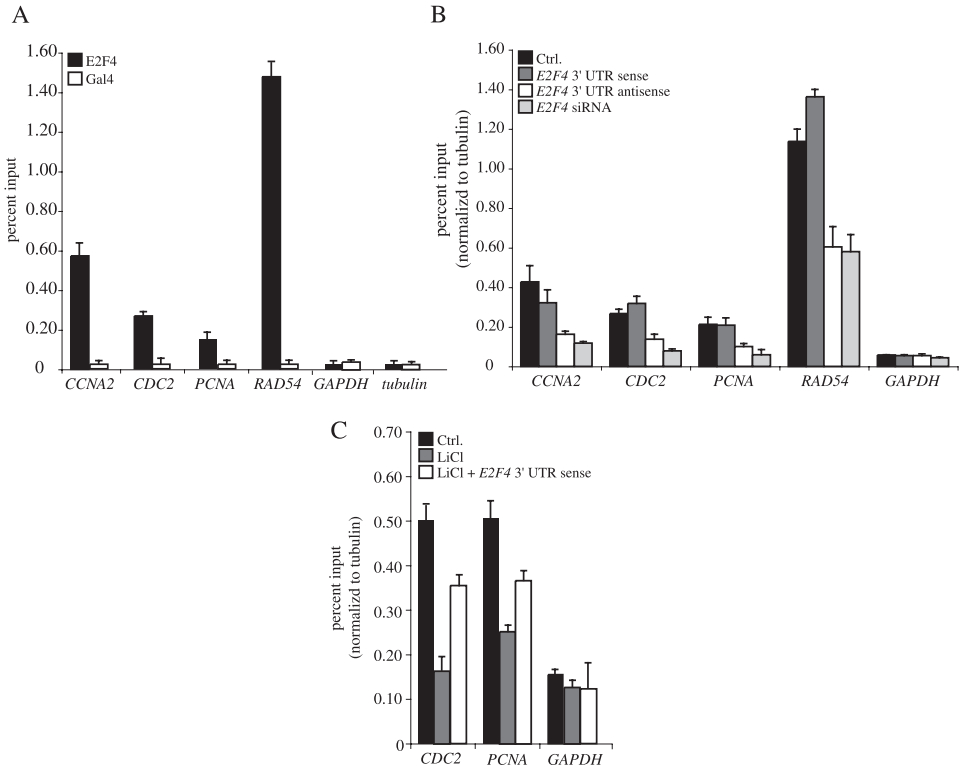

FIGURE 6. **Expression of the *E2F4* antisense transcript decreases E2F4 binding to target promoters.** *A*, E2F4 binds the promoters of target genes in HCT116 cells. Real time PCR quantitation of fragments precipitated in a ChIP assay using 3 μg of E2F4 antibodies (*black bars*) or 3 μg of Gal4 antibodies (*open bars*) as a control (*Ctrl*). Target genes are indicated on the *x* axis with *tubulin* and *GAPDH* as negative controls. The amount of DNA precipitated is indicated on the *y* axis, relative to levels obtained with input chromatin serially diluted to generate standard curves. *B*, real time PCR analysis of DNA fragments precipitated in a ChIP assay using E2F4 antibodies. HCT116 cells were not treated (control, *black bars*) or transfected with *E2F4* 3′-UTR sense (*dark gray bars*), *E2F4* 3′-UTR antisense (*open bars*), or *E2F4* siRNA (*light gray bars*) and incubated for 48 h prior to ChIP. *C*, HCT116 cells expressing the sense *E2F4* 3′-UTR were treated with 10 mM LiCl as indicated. ChIP using E2F4 antibodies was done as in *A* and *B* with promoter levels precipitated from untreated cells represented by *black bars*, those from cells treated with LiCl represented by *dark gray bars*, and cells transfected with the sense *E2F4* 3′-UTR prior to LiCl treatment represented by *open bars*. The *error bars* are S.E.

to some estimates, more than half of transcripts may have antisense correlates (11, 39, 40). The abundance of these transcripts suggests that they may provide a prevalent mechanism for gene regulation. As compared with microRNAs, whose targets are often difficult to discern, antisense RNAs are generated from the same genomic locus as their sense counterparts. Consequently, characterizing their targets should be straightforward. Surprisingly, however, relatively few examples of antisense regulation have been characterized, at least in mammalian systems. The high frequency of antisense transcription implies the existence of promoters located outside 5′-flanking regions. Indeed, internal and 3′ promoters appear to be quite abundant in studies using cap trapping strategies (10) as well as by localizing sites of TFIIB binding, which demark the locations of RNA polymerase II recruitment.[4] Antisense RNAs have been linked to multiple aspects of gene expression, including transcription, RNA processing, RNA transport, and translation, and can function in a positive or negative manner (41, 42). Much less is known about the regulation of antisense transcription. Sense and antisense transcripts are frequently expressed coordinately, but the regulation of their expression is likely to be independent, given the discrete locations of 5′ promoters and internal or 3′ transcription factor-binding sites (11). The existence of distinct regulatory elements for sense and antisense transcripts is also consistent with the idea that the antisense RNAs provide an additional level of control. It is possible, for example, that antisense transcripts could be expressed in a cell type-specific manner or that they could provide temporal aspects of regulation that cannot be achieved by transcriptional mechanisms alone. For example, antisense transcripts may be able to reduce protein expression more rapidly than what could be achieved simply by terminating transcription. Antisense transcripts could also be involved in regulating protein expression in specific subcellular domains. The finding that transcription factor-binding sites located outside known promoter regions are conserved evolutionarily is consistent with the idea that these elements are important biologically.

We developed SACO to provide an unbiased approach for unambiguously identifying transcription factor-binding sites throughout the genome (13). In particular, SACO allows identification of transcription factor-binding sites that lie outside the 5′ promoters of protein-coding genes. β-Catenin binds to DNA indirectly, and to our knowledge, genome-wide approaches such as SACO have never been applied to proteins of this nature. Consequently, we tested this approach in a screen designed to identify β-catenin genomic targets in HCT116 colorectal carcinoma cells.[3] HCT116 cells were derived from a colorectal carcinoma and resulted from a mutation that stabilizes the β-catenin protein. Thus, these cells provide a model of stimulated β-catenin signaling. In this report, we demonstrate that binding of β-catenin to a genomic region representing the 3′-UTR of the *E2F4* gene drives expression of an antisense transcript that decreases the levels of the E2F4 protein. Of note, we found that expression of a cDNA encoding the sense strand of the 3′-UTR blocked the effects of LiCl on E2F4 protein levels and promoter occupancy. This is the first nonprotein β-catenin target identified and one of a small group of antisense transcripts that has been characterized functionally. Expression of the antisense transcript leads to decreased E2F4 occupancy at endogenous E2F genomic targets, indicating that it regulates functional E2F4 pools. Because many other β-catenin genomic targets identified in our SACO library also appear to reside within regions representing the 3′-UTRs of

[4] G. Yochum, R. Cleland, V. Rajaraman, S. Impey, R. H. Goodman, and S. McWeeney, submitted for publication.

protein-coding genes,[3] it is possible that Wnt signaling induces the expression of additional antisense transcripts. This mechanism could greatly increase the repertoire of β-catenin effects.

Because of the redundancy among the various E2F proteins, establishing the specific function of a single E2F family member is difficult. Cell cycle functions of the E2F proteins have generally been examined using synchronized fibroblasts or glioblastoma cells (43–46). In this system, E2F4 has been shown to repress E2F target genes in G_0 and early G_1 by recruiting the histone deacetylase-containing complexes, p107, p130, and mSin3B (44). E2F4 has also been found to occupy the promoters of transcriptionally active genes throughout the cell cycle, suggesting that it may also be capable of recruiting coactivators in some instances (46). Fibroblasts isolated from *E2F4* knock-out mice still enter G_0 in response to serum withdrawal and cycle with normal kinetics when stimulated to re-enter the cell cycle, however, suggesting that other E2F proteins might be able to compensate for the loss of E2F4 (47, 48). In one study, for example, the effects of E2F4 could not be discerned without also eliminating expression of E2F5 (49). Thus, interrogating the occupancy of E2F4 at specific promoters is currently the most direct way to assess the effects of the *E2F4* antisense transcript. It is possible that in the context of an *E2F5* knock-out, the functional effects of the *E2F4* antisense transcript on specific cell cycle gene promoters will be more readily apparent. Such studies are currently underway.

Wnt signaling has also been studied in the colonic crypt. At the base of the crypt, Wnt is required for maintaining the proliferative capacity of the transit amplifying cells (50). As proliferative progenitors migrate toward the crypt apex, the Wnt signal ceases and the cells differentiate (50). The finding that the colonic crypts of *E2F4* knock-out mice display defects in the crypt architecture supports the idea that E2F4 protein levels must be properly maintained (47). Rather than displaying hyperplasia, as might be expected from eliminating a factor that negatively regulates cell cycle genes, the colonic crypts were reduced and lacked proliferating cells (47). It is possible that Wnt signaling, known to be critical for normal intestinal development, maintains the proper level of E2F4 protein in part through activation of the antisense transcript.

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