

Role of GAC63 in transcriptional activation mediated by β -catenin

Yong-Heng Chen^{1,2}, Catherine K. Yang², Meng Xia³, Chen-Yin Ou² and Michael R. Stallcup^{1,2,*}

¹Department of Pathology, ²Department of Biochemistry and Molecular Biology, and ³Division of Gastroenterology and Liver Diseases, University of Southern California, Los Angeles, California 90089, USA

Received October 24, 2006; Revised January 3, 2007; Accepted February 2, 2007

ABSTRACT

β -Catenin is a key mediator in the canonical Wnt signaling pathway, which plays important roles in multiple developmental processes. Inappropriate activation of this pathway leads to developmental defects and development of certain cancers. Upon Wnt signaling, β -catenin binds TCF/LEF transcription factors. The TCF/LEF- β -catenin complex then recruits a variety of transcriptional coactivators to the promoter/enhancer region of Wnt-responsive genes and activates target gene transcription. In this article, we demonstrate that GRIP1-associated coactivator 63 (GAC63), a recently identified nuclear receptor (NR) coactivator, interacts with β -catenin. The N-terminus of GAC63 is the binding site for β -catenin, whereas a C-terminal fragment of β -catenin including armadillo repeats 10–12 binds to GAC63. Over-expression of GAC63 enhanced the transcriptional activity of β -catenin, and also greatly enhanced TCF/LEF-regulated reporter gene activity in a β -catenin-dependent manner. Endogenous GAC63 was recruited to TCF/LEF-responsive enhancer elements when β -catenin levels were induced by LiCl. In addition, reduction of endogenous GAC63 level by small interfering RNA (siRNA) inhibited TCF/LEF-mediated gene transcription. Our findings reveal a new function of GAC63 in transcriptional activation of Wnt-responsive genes.

INTRODUCTION

β -Catenin is a key component of the canonical Wnt signaling pathway, which regulates a variety of developmental processes including cell growth and differentiation (1–3). Inappropriate activation of the Wnt signaling pathway is

implicated in the development of certain cancers, such as colon cancer (4–7). In the absence of Wnt ligands, cytoplasmic β -catenin levels are kept low through continuous proteasome-mediated degradation, which is controlled by a complex that contains adenomatous polyposis coli (APC), axin and glycogen synthase kinase-3 β (GSK-3). These proteins promote the phosphorylation of serine and threonine residues in the NH₂-terminal region of β -catenin, and target it for degradation by the ubiquitin-dependent proteasome degradation pathway (6,8). Upon Wnt signaling, the degradation pathway is inhibited, and subsequently β -catenin accumulates in the cytoplasm and nucleus. Nuclear β -catenin binds to transcription factors such as those belonging to the T-cell-specific transcription factor/lymphoid enhancer-binding factor (TCF/LEF) family, and activates the transcription of target genes (6), such as the genes encoding cyclin D1, c-myc, matrix metalloproteinase-7, neuronal cell adhesion molecule, interleukin-8, FGF20 and DKK1 (9–15), many of which are involved in various developmental and oncogenic processes.

In the absence of β -catenin binding, TCF/LEF transcription factors repress Wnt target gene transcription by recruiting transcription corepressors such as Groucho/TLE, CtBP and HDAC (16,17). In response to Wnt signaling, β -catenin accumulates, binds to TCF/LEF transcription factors and converts the repressor complex into a transcriptional activator complex by displacement of the corepressors from TCF/LEF and recruitment of additional transcription coactivators. These additional coactivators include histone acetyltransferase p300/CBP (18–21), the SWI/SNF ATPase subunit Brg-1 (22), p 160 coactivator GRIP1 (23), histone methyltransferase CARM1(24), CoCoA (25), Legless (26), MED12 (27), Parafibromin/Hyrax (28), TRRAP/TIP60, ISW1, MLL/Set1 (29) and the LIM protein FHL2 (30). Each transcription coactivator contributes to a signal transduction pathway which transmits the activating signal from the DNA-bound transcriptional activator protein to specific downstream targets in the transcription machinery. For example, a member of the SWI/SNF complex, Brg-1,

*To whom correspondence should be addressed. Tel: +1-323 865 3852; Fax: +1-323 865 3866; Email: stallcup@usc.edu

participates in the remodeling of chromatin conformation around the promoter by means of an ATPase activity (22). Here, we report the identification of GRIP1-associated coactivator, GAC63, as a novel coactivator for β -catenin.

GAC63, also known as HUEL, has recently been identified as a nuclear receptor (NR) coactivator (31). GAC63 interacts with the bHLH-PAS domain of p160 coactivators as well as the ligand-binding domain of some NRs, such as estrogen receptor (ER) and androgen receptor (AR). Over-expression of GAC63 enhanced transcriptional activation by NRs in a hormone-dependent manner. Although GAC63 can interact with NR directly, its coactivator function depends on the presence of a p160 coactivator with an intact N-terminal bHLH-PAS domain. Thus, it functions as a secondary coactivator in NR-mediated gene transcription. A link between GAC63 and β -catenin was first suggested by the findings that both GAC63 and β -catenin interact with androgen receptor (AR) and enhance AR function in an androgen-dependent manner (31–34). Furthermore, a variety of NR coactivators, such as CBP/p300, Brg-1, p160 coactivators, CARM1 and CoCoA, also function as coactivators in TCF/LEF-dependent gene transcription (18–25). Because of these connections, we decided to test whether GAC63 also acts as a coactivator in TCF/LEF-mediated gene transcription.

MATERIALS AND METHODS

Plasmids

The following plasmids were described previously: pSG5.HA- β -catenin, pSG5.HA-LEF1, pGEX-4T1- β -catenin, luciferase reporter plasmid pGL3OT, GK1-LUC (25); pSG5.HA-GAC63, pSG5.HA-GAC63 (1–200), pSG5.HA-GAC63 (200–370), pSG5.HA-GAC63 (370–567), pGEX-5X1-GAC63 (31). cDNA fragments encoding the indicated β -catenin amino acids were amplified by PCR and inserted into XhoI and BglII sites of pSG5.HA vector as follows: β -catenin (1–140), β -catenin (140–664), β -catenin (520–781), β -catenin (665–781).

GST pull-down assay

³⁵S-labeled full-length GAC63 and its fragments, and β -catenin and its fragments were synthesized *in vitro* by using TNT-Quick coupled transcription/translation system (Promega) according to the manufacturer's protocol. GST pull-down assays were performed as described previously (31). The amount of GST used as a control was always greater than or equal to the amount of GST fusion protein used.

Coimmunoprecipitation and immunoblotting

COS-7 cells were transfected with 3 μ g pSG5.HA- β -catenin plasmid. Cell lysates were cleared with protein A/G beads (Santa Cruz Biotechnology) for 1 h at 4°C. Two micrograms of rabbit anti-GAC63 antibody (31) or normal rabbit IgG (Santa Cruz Biotechnology) was added to the cell lysates and incubated overnight at 4°C on a rotator. Thirty microliters of protein A/G beads was

added and incubated for another 3 h. Beads were washed three times with RIPA buffer and subjected to SDS-PAGE. Blots were probed with anti- β -catenin antibody (Santa Cruz Biotechnology).

Transient transfection assay. Transient transfection assays were performed with CV-1 cells in 12-well plates as described previously (25,31). The amount of DNA transfected was held constant in all cases by using the appropriate amount of the empty vector pSG5.HA. Forty-eight hours after transfection, cells were harvested for luciferase activity assays. The results shown are the means and standard deviations of triplicate points and are representative of at least three independent experiments.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed with RKO cells treated with or without 50 mM LiCl for 45 min as described previously (31), using 2 μ g of anti- β -catenin antibody (Santa Cruz Biotechnology), 2 μ g of anti-GAC63 antibody 1bg (31) or 2 μ g of normal rabbit IgG (Santa Cruz Biotechnology). Immunoprecipitated, purified, chromosomal DNA was used for PCR amplification, using the following primers: *cyclin D1*: +128 to +486, 5'-CGGGGCAGCAGAAGCG AGA-3' (forward) and 5'-GTGAGTAGCAAAGAAACG TGG-3' (reverse); -3892 to -3649, 5'-GGTCCTCCCCG CAGTCTTC-3' (forward) and 5'-CTCTCCCCCGCAGT CAGG-3' (reverse); human *FGF20* promoter, forward 5'-CTTTCATGTGTGTCTGGGCAGGTC-3' and reverse 5'-CTTAAACCGGTCTCCTCTCAAC-3'; human *DKK1* promoter, forward 5'-GGCCACTTTGATCTCACGCG TC-3' and reverse 5'-CTGGGAACCTGGGGTGCCCTTG CC-3'. Quantitative real-time PCR reactions (QPCRs) were performed with 2 μ l (from a total of 50 μ l) of immunoprecipitated chromosomal DNA with a Stratagene Mx3000P Instrument, using the same primers as for standard PCR.

RNA interference

RNA interference experiments were performed as described previously (31) using Lipofectamine 2000 (Invitrogen). Small interfering RNA (siRNA) oligonucleotides for GAC63 and scrambled siRNA were synthesized by the USC Norris Comprehensive Cancer Center Microchemical Core Laboratory, and annealed to form duplexes. The following siRNA sequences were used: si-GAC63#1, 5'-GCUCUUGCCAGAGAGAAAAdTdT-3' (sense) and 5'-UUUUCUCUCUGGCAAGAGCdTdT-3' (antisense); si-GAC63#2, 5'-GAACCUCUCCAAGUAA GAGdTdT-3' (sense) and 5'-CUCUUACUUGGAGA GGUUCdTdT-3' (antisense); si-control with scrambled sequence (negative control siRNA having no perfect matches to known human genes), 5'-UUCUCCGAACG UGUCACAUDdTdT-3' (sense) and 5'-AUGUGACACG UUCGGAGAAAdTdT-3' (antisense). Three days after siRNA transfection, total SW480 cell RNA was extracted with Trizol reagent (Invitrogen), and subjected to reverse transcription (RT) by using iScript cDNA Synthesis Kit (Bio-Rad). Two microliters of RT product was subjected to QPCR analysis.

The primers used were as follows: human GAC63, 5'-GGCACCTTATTAGGCATGGT-3' (forward), 5'-GAATTGCCCTTACTGATGGG-3' (reverse); human cyclin D1, 5'-GCG CCC TCG GTG TCC TAC TTC AAA-3' (forward), 5'-TCG GGC CGG ATG GAG TTG TCG-3' (reverse); human β -actin, 5'-ACCCCATCG AGCACGGCATCG-3' (forward), 5'-GTCACCGGAGT CCATCACGATG-3' (reverse).

RESULTS

Interaction of GAC63 with β -catenin

Both β -catenin and GAC63 bind to AR and enhance transcriptional activation by AR (31–34). We therefore tested whether GAC63 can interact with β -catenin *in vitro* using GST pull-down assays. GAC63 synthesized *in vitro* specifically and efficiently bound to GST- β -catenin, while GST alone was unable to pull down GAC63 (Figure 1A). These data suggest that GAC63 binds β -catenin directly *in vitro*.

To test for binding *in vivo*, we performed coimmunoprecipitation assay. COS-7 cells were transiently transfected with β -catenin, and cell lysates were immunoprecipitated by anti-GAC63 antibody followed by immunoblotting analysis with anti- β -catenin antibody. β -catenin was coimmunoprecipitated by antibody against GAC63, but not by normal rabbit IgG (Figure 1B). Thus, these results indicate that GAC63 interacts with β -catenin *in vivo*.

We also tested whether GAC63 binds to LEF1 in GST pull-down assays. β -catenin synthesized *in vitro* bound

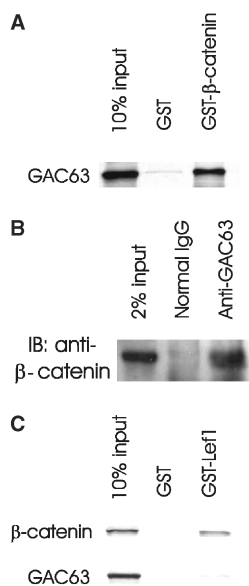


Figure 1. GAC63 interacts with β -catenin *in vitro* and *in vivo*. (A) In GST pull-down assays, GST- β -catenin fusion protein was bound to beads and incubated with *in vitro* synthesized and 35 S-labeled GAC63. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. (B) COS-7 cells were transfected with 3 μ g pSG5. HA- β -catenin plasmid. The cell extracts were immunoprecipitated with anti-GAC63 antibody or normal rabbit IgG. The immunoprecipitated proteins were detected by immunoblot with anti- β -catenin antibody. (C) β -catenin and GAC63 were translated *in vitro* and tested for binding to GST or GST-LEF1.

strongly and specifically to GST-LEF1 (Figure 1C, upper panel), but GAC63 synthesized *in vitro* did not (Figure 1C, lower panel). These results suggest that GAC63 cannot interact directly with LEF1 *in vitro*.

Mapping of interaction domains in GAC63 and β -catenin

GAC63 has a zinc-finger-like motif at the N-terminus, two leucine-zipper-like motifs at the central region and an LXXLL motif and an acidic region at the C-terminus (35) (Figure 2A). To narrow down the β -catenin interaction domain in GAC63, a series of GAC63 deletion mutants were synthesized *in vitro*, and tested for their binding to GST- β -catenin. Among them, a fragment of the N-terminal region (amino acids 1–200) efficiently and specifically bound GST- β -catenin, but fragments of the central region (amino acids 200–370) and the C-terminus (amino acids 370–567) bound weakly or not at all (Figure 2B). Taken together, these results suggest that β -catenin directly binds to the N-terminus of GAC63.

β -catenin is composed of three domains: a regulatory N-terminal region followed by 12 armadillo (Arm) repeats and a C-terminal transactivation domain (36) (Figure 2A). In order to determine the domain of β -catenin that interacts with GAC63, we performed GST pull-down

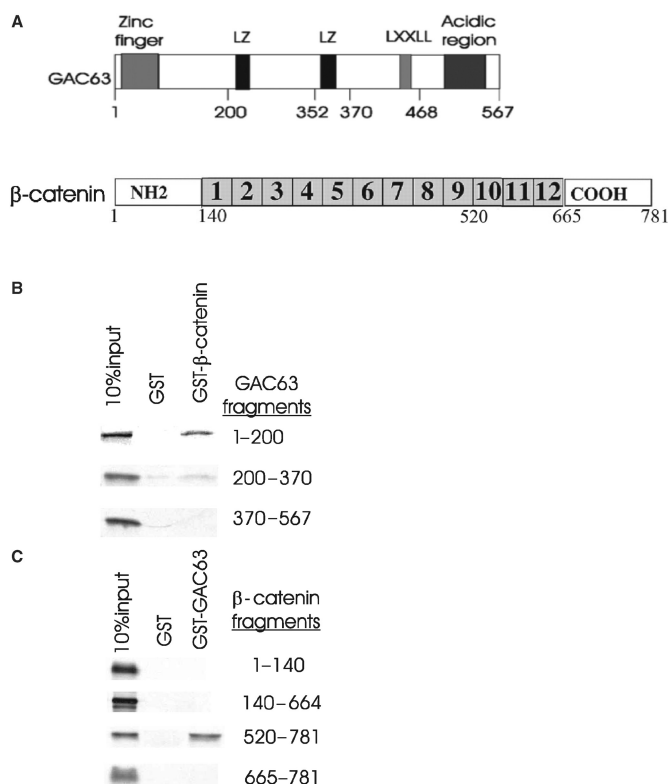


Figure 2. Determination of interaction domains of GAC63 and β -catenin. (A) Functional domains of GAC63 and β -catenin. Numbers beneath each diagram indicate amino acids. LXXLL, leucine-rich motif; LZ, leucine zipper-like motif. Numbered segments of β -catenin represent armadillo repeats. (B) The indicated GAC63 fragments were synthesized *in vitro* and tested in GST pull-down assays for binding to GST or GST- β -catenin. (C) The *in vitro* synthesized β -catenin fragments were tested in GST pull-down assays for binding to GST or GST-GAC63.

assays using a series of β -catenin deletion mutants. GST-GAC63 specifically bound a β -catenin fragment consisting of Arm repeats 10–12 and the C-terminal activation domain. No interaction was seen with the N-terminus, Arm repeats 1–12 or the C-terminus alone (Figure 2C). These results suggest that a C-terminal fragment of β -catenin consisting of Arm repeats 10–12 and the C-terminal activation domain binds GAC63.

GAC63 functions as a β -catenin dependent coactivator in TCF/LEF-mediated transcription

GAC63 is a secondary coactivator in NR-mediated gene transcription. Although it can bind NR directly as well as the p160 NR coactivator GRIP1, its coactivator function depends on the presence of GRIP1 (31). On the other hand, β -catenin can bind directly to TCF/LEF proteins and activate TCF/LEF-mediated gene transcription (6). Since GAC63 interacts with β -catenin, we tested whether GAC63 also functions as a coactivator in the TCF/LEF-mediated pathway in transient transfection assays using the luciferase reporter plasmid pGL3OT, which contains TCF/LEF-responsive elements. As expected, β -catenin enhanced the reporter gene activity by 6-fold (Figure 3A, assay 4). Over-expression of GAC63 alone had little effect on reporter gene activity (Figure 3A, assays 2 and 3). However, over-expression of GAC63 and β -catenin together dramatically and synergistically increased reporter gene expression up to 60-fold (Figure 3A, assays 5 and 6). The over-expression of GAC63 had no effect on the level of β -catenin expressed (data not shown). In another control experiment, the mutant reporter plasmid pGL3OF, containing mutant TCF/LEF-responsive elements, was not activated by either β -catenin alone or β -catenin plus GAC63 (data not shown). These results, along with the inability of GAC63 to bind directly to LEF1, suggest that GAC63 functions as a secondary coactivator in TCF/LEF-mediated transcriptional activation, i.e. the coactivator function of GAC63 depends on the presence of β -catenin.

To further evaluate this hypothesis, we tested the effect of GAC63 on the transcriptional activity of LEF1 fused to the Gal4 DNA-binding domain (DBD), in the absence or presence of β -catenin. Gal4DBD-LEF1 fusion protein itself only had a weak autonomous transactivation activity to activate transcription of a reporter gene containing Gal4 response elements, and β -catenin strongly enhanced this activity (Figure 3B, assays 5 and 6). In the absence of β -catenin, GAC63 had little effect on the Gal4-responsive reporter gene expression (Figure 3B, assay 7); however, in the presence of β -catenin, GAC63 acted synergistically with β -catenin to enhance the reporter gene expression (Figure 3B, assay 8). These data further support the idea that GAC63 enhances LEF1 activity through β -catenin.

Since GAC63 enhances LEF1 transcriptional activity through β -catenin, we then tested whether GAC63 could affect the transcriptional activity of β -catenin. In transiently transfected CV1 cells, Gal4DBD- β -catenin fusion protein showed modest autonomous transactivation

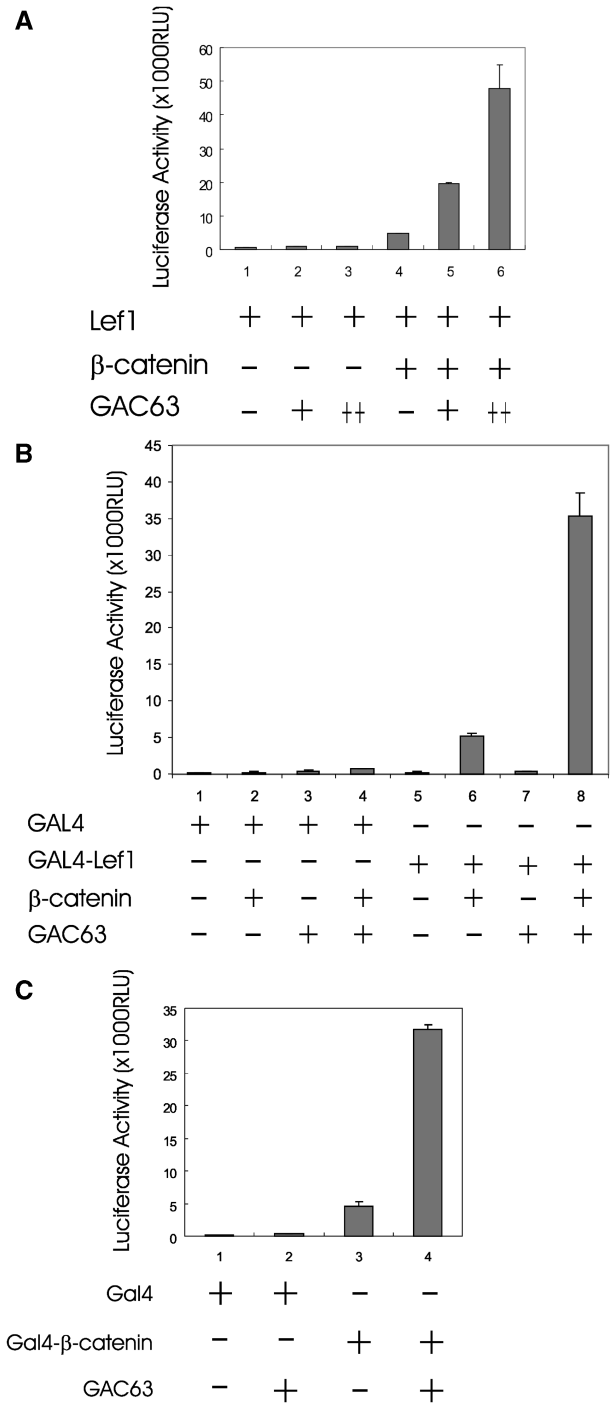


Figure 3. GAC63 functions as a β -catenin dependent coactivator in TCF/LEF-mediated transcription. (A) CV-1 cells were transfected in 12-well plates with 200 ng pGL3OT reporter plasmid; 10 ng pSG5.HA-LEF1; 100 ng pSG5.HA- β -catenin; and 200 ng (+) or 400 ng (++) pSG5.HA-GAC63 as indicated. Results shown are representative of three independent experiments. (B) CV-1 cells were transfected with 200 ng of GK1-LUC reporter plasmid, 200 ng of plasmid encoding Gal4 DBD or Gal4-LEF1, either 400 ng of GAC63 expression plasmid, or 200 ng of β -catenin, or both. The luciferase activity results shown are representative of three independent experiments. (C) CV-1 cells were transfected with 200 ng of GK1-LUC reporter plasmid, 200 ng of plasmid encoding Gal4 DBD or Gal4- β -catenin, and 400 ng of GAC63 expression plasmid as indicated. The luciferase activity results shown are representative of three independent experiments.

activity to activate transcription of the Gal4-responsive reporter gene (Figure 3C, assay 3). This activity was further enhanced (up to 7-fold) by co-expression of GAC63 (Figure 3C, assay 4). Thus, GAC63 enhances the transcriptional activity of β -catenin.

Endogenous GAC63 is recruited to TCF/LEF-responsive enhancer elements upon LiCl treatment

To test whether endogenous GAC63 is specifically recruited to endogenous TCF/LEF-responsive enhancer elements *in vivo*, we employed chromatin immunoprecipitation (ChIP) assays to examine the *cyclin D1* promoter in RKO colon cancer cells upon the addition of LiCl, a GSK-3 β inhibitor. To determine the optimal concentration of LiCl to inhibit the degradation of β -catenin, RKO cells were treated with different concentrations of LiCl for 45 min. As shown in Figure 4A, β -catenin protein levels increased upon the addition of LiCl in a dose-dependent manner. Thus, we decided to treat RKO cells with 50 mM LiCl for 45 min in ChIP assays. As expected, β -catenin was recruited to *cyclin D1* promoter region after a 45-min LiCl treatment. Similarly, endogenous GAC63 was also recruited to this promoter upon the addition of LiCl (Figure 4B). Normal IgG served as a negative control, and the input chromatin levels from LiCl-treated or untreated cells were equivalent. The recruitment of β -catenin and GAC63 was specific for the promoter region of *cyclin D1* gene, because PCRs with primers for a region 3.6 kb upstream which lacked TCF/LEF-binding sites failed to produce a signal from the same immunoprecipitated chromatin fraction. QPCR analysis confirmed the recruitment of β -catenin and GAC63 to the promoter region of *cyclin D1* upon LiCl treatment (Figure 4C). The same immunoprecipitated chromatin fraction was also tested by PCR using primers to amplify the promoter regions of two other Wnt target genes, *FGF20* and *DKK1* (15). Both β -catenin and GAC63 were recruited to these promoter regions upon LiCl treatment (Figure 4D).

Endogenous GAC63 is important for efficient transcriptional activation by LEF1

Over-expression of GAC63 enhances LEF1-responsive reporter gene expression (Figure 3A). To test for a physiological role of endogenous GAC63 in the process of transcriptional activation by LEF1, we examined the effect of reduced endogenous GAC63 on LEF1-regulated *cyclin D1* gene expression in SW480 colon cancer cells. These cells contain a mutation in the adenomatous polyposis coli protein, which regulates β -catenin degradation. Thus, these cells have elevated levels of wild-type β -catenin, and therefore they have elevated expression of *cyclin D1* mRNA and protein (25). Two different siRNAs targeting the human GAC63 sequence specifically reduced the level of endogenous GAC63 mRNA (Figure 5A, upper panel) and protein (Figure 5B, upper panel), while the non-specific control siRNA did not. The expression of the LEF1- and β -catenin-regulated *cyclin D1* gene was inhibited by 40–50% in the presence of siRNA against GAC63, but was not affected by an equivalent amount of control

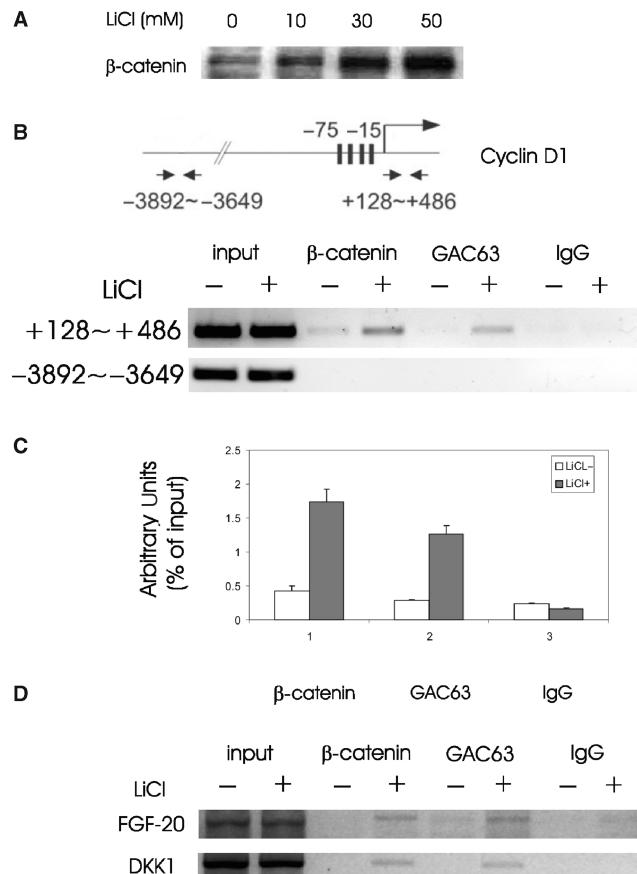


Figure 4. Endogenous GAC63 is recruited to TCF/LEF-responsive *cyclin D1* promoter region upon LiCl treatment. (A) RKO cells were treated with different concentrations of LiCl for 45 min. Cell extracts were tested by immunoblotting using anti- β -catenin antibody. (B) RKO cells were grown with or without 50 mM LiCl for 45 min and analyzed by chromatin immunoprecipitation assays, using the indicated antibodies for immunoprecipitation. The immunoprecipitated DNA was amplified by PCR using primers to amplify the promoter region of *cyclin D1* gene and a region 3.6 kb upstream. Results shown are representative of two independent experiments. (C) QPCR analysis of immunoprecipitated RKO cell chromosomal DNA using primers for the TCF/LEF binding region of *cyclin D1* gene was performed with 2 μ l samples. The results are shown as percentage of input, are the mean and S.D. from triplicate reactions, and are representative of two independent experiments. (D) PCR analysis of immunoprecipitated RKO cell chromosomal DNA using primers for the promoter regions of *FGF20* and *DKK1* genes. Results shown are representative of two independent experiments.

siRNA with scrambled sequence (Figure 5A, lower panel). The effect on *cyclin D1* gene expression was specific, since the results shown are normalized to the level of β -actin transcripts. Thus, although many different coactivators are involved in mediating transcriptional activation by LEF1 and β -catenin, endogenous GAC63 is important for efficient induction of the LEF1- and β -catenin-regulated *cyclin D1* gene.

DISCUSSION

The Wnt signaling pathway plays important roles in multiple developmental processes and cancer (1–7). Upon Wnt signaling, β -catenin associates with TCF/LEF

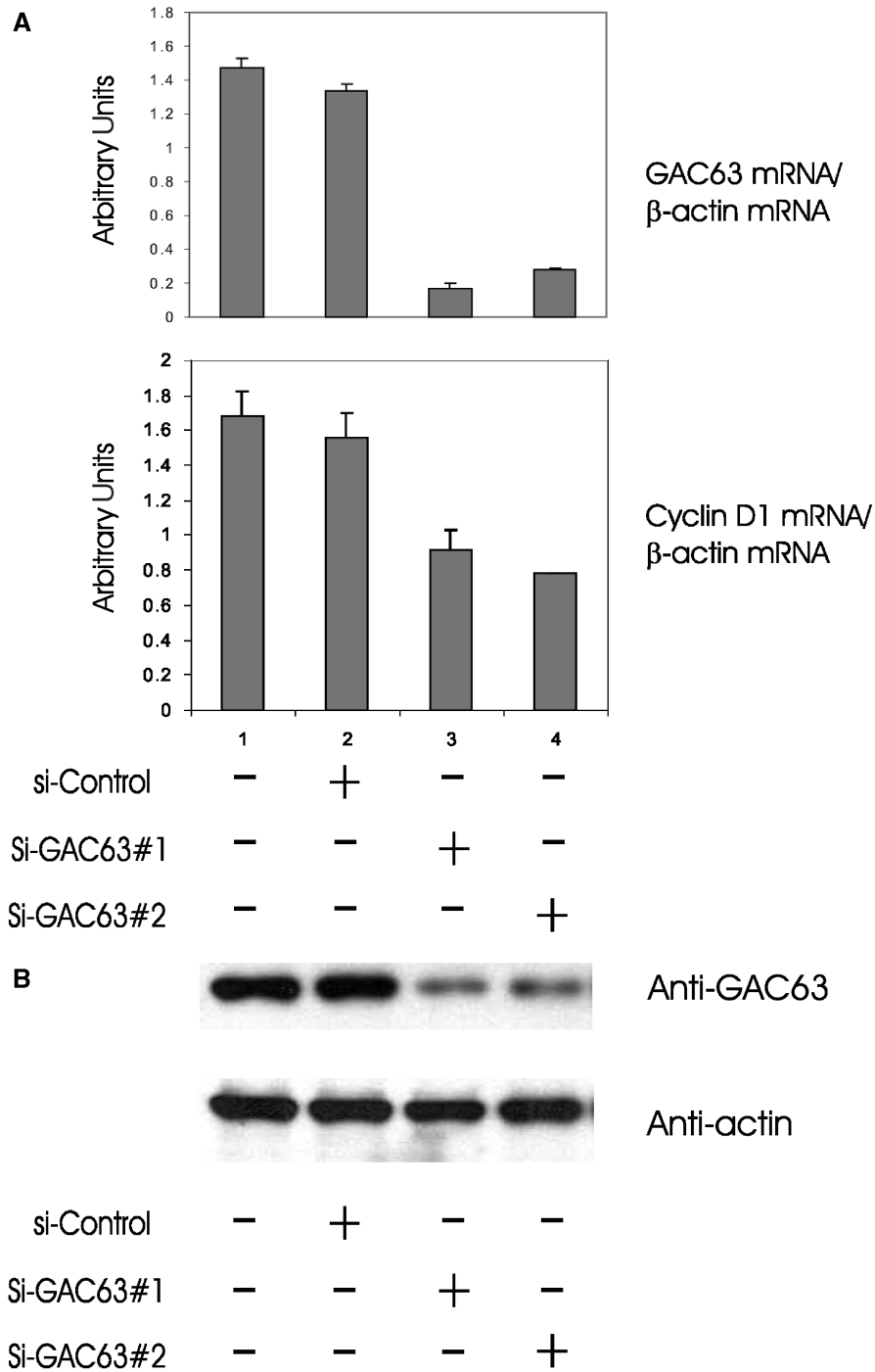


Figure 5. Endogenous GAC63 is important for efficient transcriptional activation by LEF1. (A) SW480 cells were transfected with siRNA duplex against GAC63 or a non-specific control siRNA duplex. After 72 h, cells were harvested for either immunoblots or total RNA preparation. mRNA was analyzed by RT-QPCR to measure the levels of human GAC63, cyclin D1 and β -actin transcripts. Results shown for GAC63 and cyclin D1 transcripts were normalized by the levels of β -actin transcripts and are representative of three independent experiments. A paired, two-tailed *t*-test performed on the values from the three experiments indicated that the siRNA against GAC63 caused significant decreases in the expression levels of cyclin D1 mRNA ($P=0.0004$ for 1 and $P=0.0008$ for #2). For the three experiments, the mean decrease and 95% confidence intervals were $85 \pm 5\%$ (#1) and $85 \pm 7\%$ (#2) for GAC63 mRNA, and $46 \pm 12\%$ (#1) and $51 \pm 7\%$ (#2) for cyclin D1 mRNA. (B) Cell extracts were also tested by immunoblotting using anti-GAC63 and anti-actin antibodies.

transcription factors and recruits a variety of transcriptional coactivators to the promoter/enhancer regions of Wnt-responsive genes. Here we report that GAC63 is a novel binding partner and coactivator of β -catenin.

GAC63 binds β -catenin *in vitro* and *in vivo*, but does not bind directly to LEF1 (Figure 1). Over-expression of GAC63 enhanced TCF/LEF-mediated transcription in a β -catenin-dependent manner (Figure 3). Taken together,

our data suggest that GAC63 functions as a secondary coactivator in TCF/LEF-mediated transcriptional activation, that is, its coactivator function depends on the presence of β -catenin. Moreover, upon LiCl treatment, endogenous GAC63 is recruited to the promoter regions of endogenous Wnt target genes, such as *cyclin D1*, *FGF20* and *DKK1* (Figure 4). In addition, reduction of endogenous GAC63 by siRNA inhibited the TCF/LEF- and β -catenin-mediated expression of the *cyclin D1* gene by 40–50% in a cell line with constitutively high levels of β -catenin and cyclin D1 expression (Figure 5). The lack of complete inhibition by siRNA could be due to residual GAC63, or the compensatory effects by other transcription coactivators, such as CBP/p300, Brg-1, GRIP1, CARM1 and CoCoA, which also bind to β -catenin and serve as secondary coactivators for TCF/LEF transcription factors. Furthermore, GAC63 could be recruited to other regulatory sites on the *cyclin D1* promoter in addition to the TCF/LEF-binding site; therefore, it is possible that the reduced cyclin D1 expression observed when GAC63 levels are reduced by siRNA could be due to reduced GAC63 interactions at the TCF/LEF site and other regulatory sites. While we do not know whether GAC63 is required for β -catenin-mediated up-regulation of cyclin D1 expression in all regulatory settings, our data as a whole make a compelling case that GAC63 plays an important role in β -catenin- and TCF/LEF-mediated transcription. We therefore conclude that GAC63 is a physiological component of TCF/LEF- and β -catenin-mediated transactivation, and is important for optimal transcriptional activation mediated by β -catenin and TCF/LEF in at least some cases.

In previous studies, GAC63 has been shown to function as a secondary coactivator for NRs by cooperating with p160 coactivators (31). The current study shows that GAC63 functions as a secondary coactivator for another class of transcription factor, TCF/LEF, by cooperating with β -catenin. GAC63 also works as a primary coactivator for the aryl hydrocarbon receptor (AHR), i.e. it binds directly to AHR (37). Thus, GAC63 functions as a general transcriptional coactivator for multiple signaling pathways. However, we cannot rule out the possibility that GAC63 could also act as corepressor in certain conditions. A number of proteins have been shown to act as either coactivator or corepressor in a promoter-specific manner. For example, members of the p160 coactivators, such as GRIP1, function as glucocorticoid receptor (GR) coactivators. However, at the promoters of certain GR target genes that are repressed by hormone-activated GR, such as the human collagenase-3 gene and osteocalcin gene, GRIP1 acts as a GR corepressor (38).

GAC63 functions as a coactivator in multiple signaling pathways; however, the mechanisms by which GAC63 contributes to transcriptional activation are yet to be established. GAC63 has an N-terminal region containing a zinc-finger-like motif, a central domain containing two leucine-zipper-like motifs, and a C-terminal region with a LXXLL motif and an acidic region (35). One region of the GAC63 protein (amino acids 127–196) was shown to have sequence similarity (34%) to the DNA-binding

domain (amino acids 21–95) of the XPA DNA repair protein (SWISS-PROT accession no. Q64029) (39). Another GAC63 region (amino acids 239–533) shares homology with members of the zinc transporter family. In fragment studies, the N-terminus and central region of GAC63 both bind GRIP1, while only the N-terminus binds β -catenin. When fused to Gal4 DNA-binding domain, the C-terminus of GAC63 showed weak autonomous transcriptional activation activity (31). GAC63 also contains several nuclear localization signals and a nuclear-export-like signal; the human ortholog of GAC63, HUEL, has been shown to undergo nuclear translocation during S phase (39).

β -Catenin has been shown to be a coactivator for TCF/LEF transcription factors as well as androgen receptor. This provides a mechanism for crosstalk between the Wnt and AR signaling pathways. In addition, these two pathways share a number of transcriptional coactivators, such as CBP/p300 (18–21), Brg-1 (22), p160 coactivators (23), CARM1 (24) and CoCoA (25). GAC63 provides yet another example of a transcriptional coactivator that is involved in both signaling pathways. Furthermore, members of the NR superfamily, such as androgen receptor (AR), thyroid hormone receptor (TR) and retinoic acid receptor (RAR), have been shown to interfere with the Wnt signaling pathway (32,40–45). The antagonistic effect by AR could be due to the competition for common transcriptional coactivators, in addition to other proposed mechanisms, such as increased degradation of β -catenin (40,46).

In our study, GAC63 bound to a β -catenin fragment consisting of Arm repeats 10–12 and the C-terminal activation domain. Several other proteins have been reported to bind to a similar β -catenin subdomain, and contribute to transcriptional activation. These proteins include CBP/p300, MED12, Parafibromin/Hyrax, TRRAP/TIP60, ISW1 and MLL/Set1 (18,21,27–29,47). It remains to be determined whether these coactivators can bind to β -catenin at the same time and function synergistically as coactivators, or whether some of them may compete for binding and inhibit each other's function or play different roles on various promoters. For example, although CBP and p300 are closely related, they play opposite roles on the expression of some β -catenin target genes, such as survivin (48). The small molecule ICG-001, which blocks the CBP– β -catenin interaction but not the p300– β -catenin interaction, inhibits survivin gene expression. In the absence of ICG-001, CBP is recruited to the survivin promoter, and promotes survivin gene expression; however, in the presence of ICG-001, there is less CBP on the survivin promoter but more p300 instead, which leads to concomitant recruitment of repressive elements and results in transcription repression.

Inappropriate activation of the Wnt signaling pathway occurs in many types of cancer, and is caused by mutations of many of the Wnt signaling components (4–7). The two genes found most frequently mutated are adenomatous polyposis coli (APC) and β -catenin. These mutations generally lead to stabilization of β -catenin, and an over-activation of the Wnt signaling pathway. This over-activation is thought in part to promote oncogenesis

through over-expression of Wnt-responsive genes, such as *c-myc* and *cyclin D1* (9–11). Since GAC63 cooperates with β -catenin to activate Wnt-responsive gene expression, GAC63 might play a role in the oncogenic processes. Furthermore, in DNA microarray studies, GAC63 (*C4orf1*) over-expression has been shown to be associated with lymph node metastasis of breast cancer (49). In addition, a distinct alternative transcript encoding GAC63 with a modified and truncated C-terminus was detected in the HT-1080 fibrosarcoma, G-401 Wilms tumor and SiHa cervical carcinoma cell lines (35). These findings indicate that GAC63 might be involved in the development of cancer. The role of GAC63 in oncogenesis, and the mechanisms by which GAC63 contributes to oncogenesis are currently under investigation in our laboratory.

ACKNOWLEDGEMENTS

We thank Daniel Gerke and Kelly Chang (University of Southern California) for expert technical assistance. This work was supported by grant DK43093 to M.R.S. from the U.S. National Institutes of Health. Y.H.C. was supported in part by a predoctoral fellowship from the University of Southern California/Norris Breast Cancer Research Training Program. Funding to pay the Open Access publication charge was provided by grant DK43093 to M.R.S. from the United States National Institutes of Health and by the University of Southern California.

Conflict of interest statement. None declared.

REFERENCES

- Moon, R.T., Brown, J.D. and Torres, M. (1997) WNTs modulate cell fate and behavior during vertebrate development. *Trends Genet.*, **13**, 157–162.
- Miller, J.R., Hocking, A.M., Brown, J.D. and Moon, R.T. (1999) Mechanism and function of signal transduction by the Wnt/ β -catenin and Wnt/ Ca^{2+} pathways. *Oncogene*, **18**, 7860–7872.
- Cadigan, K.M. and Nusse, R. (1997) Wnt signaling: a common theme in animal development. *Genes Dev.*, **11**, 3286–3305.
- Polakis, P. (2000) Wnt signaling and cancer. *Genes Dev.*, **14**, 1837–1851.
- Reya, T. and Clevers, H. (2005) Wnt signalling in stem cells and cancer. *Nature*, **434**, 843–850.
- Moon, R.T., Kohn, A.D., Ferrari, G.V.D. and Kaykas, A. (2004) Wnt and β -catenin signalling: diseases and therapies. *Nat. Rev. Genet.*, **5**, 691–701.
- Giles, R.H., van Es, J.H. and Clevers, H. (2003) Caught up in a Wnt storm: Wnt signaling in cancer. *Biochim. Biophys. Acta Rev. Cancer*, **1653**, 1–24.
- Aberle, H., Bauer, A., Stappert, J., Kispert, A. and Kemler, R. (1997) β -Catenin is a target for the ubiquitin-proteasome pathway. *EMBO J.*, **16**, 3797–3804.
- He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B. and Kinzler, K.W. (1998) Identification of c-MYC as a target of the APC pathway. *Science*, **281**, 1509–1512.
- Tetsu, O. and McCormick, F. (1999) β -Catenin regulates expression of cyclin D1 in colon carcinoma cells. *Nature*, **398**, 422–426.
- Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R. and Ben Ze'ev, A. (1999) The cyclin D1 gene is a target of the β -catenin/LEF-1 pathway. *Proc. Natl. Acad. Sci. USA*, **96**, 5522–5527.
- Brabletz, T., Jung, A., Dag, S., Hlubek, F. and Kirchner, T. (1999) β -Catenin regulates the expression of the matrix metalloproteinase-7 in human colorectal cancer. *Am. J. Pathol.*, **155**, 1033–1038.
- Conacci-Sorrell, M.E., Ben Yedidia, T., Shtutman, M., Feinstein, E., Einat, P. and Ben Ze'ev, A. (2002) Nr-CAM is a target gene of the β -catenin/LEF-1 pathway in melanoma and colon cancer and its expression enhances motility and confers tumorigenesis. *Genes Dev.*, **16**, 2058–2072.
- Levy, L., Neuveut, C., Renard, C.A., Charneau, P., Branchereau, S., Gauthier, F., Van Nhieu, J.T., Cherqui, D., Petit-Bertron, A.F. et al. (2002) Transcriptional activation of interleukin-8 by β -catenin-Tcf4. *J. Biol. Chem.*, **277**, 42386–42393.
- Chamorro, M.N., Schwartz, D.R., Vonica, A., Brivanlou, A.H., Cho, K.R. and Varmus, H.E. (2005) FGF-20 and DKK1 are transcriptional targets of β -catenin and FGF-20 is implicated in cancer and development. *EMBO J.*, **24**, 73–84.
- Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O. and Clevers, H. (1998) The Xenopus Wnt effector XTcf-3 interacts with Groucho-related transcriptional repressors. *Nature*, **395**, 608–612.
- Brannon, M., Brown, J.D., Bates, R., Kimelman, D. and Moon, R.T. (1999) XctBP is a XTcf-3 co-repressor with roles throughout Xenopus development. *Development*, **126**, 3159–3170.
- Hecht, A., Vleminckx, K., Stemmler, M.P., van Roy, F. and Kemler, R. (2000) The p300/CBP acetyltransferases function as transcriptional coactivators of β -catenin in vertebrates. *EMBO J.*, **19**, 1839–1850.
- Miyagishi, M., Fujii, R., Hatta, M., Yoshida, E., Araya, N., Nagafuchi, A., Ishihara, S., Nakajima, T. and Fukamizu, A. (2000) Regulation of Lef-mediated transcription and p53-dependent pathway by associating β -catenin with CBP/p300. *J. Biol. Chem.*, **275**, 35170–35175.
- Sun, Y., Kolligs, F.T., Hottiger, M.O., Mosavin, R., Fearon, E.R. and Nabel, G.J. (2000) Regulation of β -catenin transformation by the p300 transcriptional coactivator. *Proc. Natl. Acad. Sci. USA*, **97**, 12613–12618.
- Takemaru, K.I. and Moon, R.T. (2000) The transcriptional coactivator CBP interacts with β -catenin to activate gene expression. *J. Cell. Biol.*, **149**, 249–254.
- Barker, N., Hurlstone, A., Musisi, H., Miles, A., Bienz, M. and Clevers, H. (2001) The chromatin remodelling factor Brg-1 interacts with β -catenin to promote target gene activation. *EMBO J.*, **20**, 4935–4943.
- Li, H., Kim, J.H., Koh, S.S. and Stallcup, M.R. (2004) Synergistic effects of coactivators GRIP1 and β -catenin on gene activation: cross-talk between androgen receptor and Wnt signaling pathways. *J. Biol. Chem.*, **279**, 4212–4220.
- Koh, S.S., Li, H., Lee, Y.H., Widelitz, R.B., Chuong, C.M. and Stallcup, M.R. (2002) Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and β -catenin with two different classes of DNA-binding transcriptional activators. *J. Biol. Chem.*, **277**, 26031–26035.
- Yang, C.K., Kim, J.H., Li, H. and Stallcup, M.R. (2006) Differential use of functional domains by coiled-coil coactivator in its synergistic coactivator function with β -catenin or GRIP1. *J. Biol. Chem.*, **281**, 3389–3397.
- Kramps, T., Peter, O., Brunner, E., Nellen, D., Froesch, B., Chatterjee, S., Murone, M., Zullig, S. and Basler, K. (2002) Wnt/Wingless signaling requires BCL9/legless-mediated recruitment of pygopus to the nuclear β -catenin-TCF complex. *Cell*, **109**, 47–60.
- Kim, S., Xu, X., Hecht, A. and Boyer, T.G. (2006) Mediator is a transducer of Wnt/ β -catenin signaling. *J. Biol. Chem.*, **281**, 14066–14075.
- Mosimann, C., Hausmann, G. and Basler, K. (2006) Parafibromin/hyrax activates Wnt/Wg target gene transcription by direct association with β -catenin/armadillo. *Cell*, **125**, 327–341.
- Sierra, J., Yoshida, T., Joazeiro, C.A. and Jones, K.A. (2006) The APC tumor suppressor counteracts β -catenin activation and H3K4 methylation at Wnt target genes. *Genes Dev.*, **20**, 586–600.

30. Wei, Y., Renard, C.A., Labalette, C., Wu, Y., Levy, L., Neuveut, C., Prieur, X., Flajolet, M., Prigent, S. *et al.* (2003) Identification of the LIM Protein FHL2 as a Coactivator of beta-catenin. *J. Biol. Chem.*, **278**, 5188–5194.
31. Chen, Y.H., Kim, J.H. and Stallcup, M.R. (2005) GAC63, a GRIP1-dependent nuclear receptor coactivator. *Mol. Cell Biol.*, **25**, 5965–5972.
32. Pawlowski, J.E., Ertel, J.R., Allen, M.P., Xu, M., Butler, C., Wilson, E.M. and Wierman, M.E. (2002) Liganded androgen receptor interaction with beta-catenin. nuclear co-localization and modulation of transcriptional activity in neuronal cells. *J. Biol. Chem.*, **277**, 20702–20710.
33. Yang, F., Li, X., Sharma, M., Sasaki, C.Y., Longo, D.L., Lim, B. and Sun, Z. (2002) Linking beta-catenin to androgen-signaling pathway. *J. Biol. Chem.*, **277**, 11336–11344.
34. Truica, C.I., Byers, S. and Gelmann, E.P. (2000) {beta}-Catenin affects androgen receptor transcriptional activity and ligand specificity. *Cancer Res.*, **60**, 4709–4713.
35. Sim, D.L.C. and Chow, V.T.K. (1999) The novel human HUEL (C4orf1) gene maps to chromosome 4p12-p13 and encodes a nuclear protein containing the nuclear receptor interaction motif. *Genomics*, **59**, 224–233.
36. Willert, K. and Nusse, R. (1998) Beta-catenin: a key mediator of Wnt signaling. *Curr. Opin. Genet. Dev.*, **8**, 95–102.
37. Chen, Y.H., Beischlag, T.V., Kim, J.H., Perdew, G.H. and Stallcup, M.R. (2006) Role of GAC63 in transcriptional activation mediated by the aryl hydrocarbon receptor. *J. Biol. Chem.*, **281**, 12242–12247.
38. Rogatsky, I., Luecke, H.F., Leitman, D.C. and Yamamoto, K.R. (2002) Alternate surfaces of transcriptional coregulator GRIP1 function in different glucocorticoid receptor activation and repression contexts. *Proc. Natl. Acad. Sci. USA*, **99**, 16701–16706.
39. Sim, D.L.C., Yeo, W.M. and Chow, V.T.K. (2002) The novel human HUEL (C4orf1) protein shares homology with the DNA-binding domain of the XPA DNA repair protein and displays nuclear translocation in a cell cycle-dependent manner. *Int. J. Biochem. Cell Biol.*, **34**, 487–504.
40. Mulholland, D.J., Dedhar, S., Coetzee, G.A. and Nelson, C.C. (2005) Interaction of nuclear receptors with the Wnt/{beta}-catenin/Tcf signaling axis: Wnt you like to know? *Endocr. Rev.*, **26**, 898–915.
41. Song, L.N., Herrell, R., Byers, S., Shah, S., Wilson, E.M. and Gelmann, E.P. (2003) {beta}-Catenin binds to the activation function 2 region of the androgen receptor and modulates the effects of the N-terminal domain and TIF2 on ligand-dependent transcription. *Mol. Cell Biol.*, **23**, 1674–1687.
42. Mulholland, D.J., Read, J.T., Rennie, P.S., Cox, M.E. and Nelson, C.C. (2003) Functional localization and competition between the androgen receptor and T-cell factor for nuclear beta-catenin: a means for inhibition of the Tcf signaling axis. *Oncogene*, **22**, 5602–5613.
43. Shah, S., Hecht, A., Pestell, R. and Byers, S.W. (2003) Trans-repression of {beta}-catenin activity by nuclear receptors. *J. Biol. Chem.*, **278**, 48137–48145.
44. Easwaran, V., Pishvaian, M., Salimuddin, and Byers, S. (1999) Cross-regulation of [beta]-catenin-LEF/TCF and retinoid signaling pathways. *Curr. Biol.*, **9**, 1415–1419.
45. Miller, L.D., Park, K.S., Guo, Q.M., Alkharouf, N.W., Malek, R.L., Lee, N.H., Liu, E.T. and Cheng, S.Y. (2001) Silencing of Wnt signaling and activation of multiple metabolic pathways in response to thyroid hormone-stimulated cell proliferation. *Mol. Cell Biol.*, **21**, 6626–6639.
46. Natsume, H., Sasaki, S., Kitagawa, M., Kashiwabara, Y., Matsushita, A., Nakano, K., Nishiyama, K., Nagayama, K., Misawa, H. *et al.* (2003) [beta]-Catenin/Tcf-1-mediated transactivation of cyclin D1 promoter is negatively regulated by thyroid hormone. *Biochem. Biophys. Res. Commun.*, **309**, 408–413.
47. Stadel, R., Hoffmans, R. and Basler, K. (2006) Transcription under the control of nuclear Arm/beta-catenin. *Curr. Biology*, **16**, R378–R385.
48. Ma, H., Nguyen, C., Lee, K.S. and Kahn, M. (2005) Differential roles for the coactivators CBP and p300 on TCF/beta-catenin-mediated survivin gene expression. *Oncogene*, **24**, 3619–3631.
49. Huang, E., Cheng, S.H., Dressman, H., Pittman, J., Tsou, M.H., Horng, C.F., Bild, A., Iversen, E.S., Liao, M. *et al.* (2003) Gene expression predictors of breast cancer outcomes. *Lancet*, **361**, 1590–1596.