THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 3, pp. 1755–1764, January 20, 2006

HIC-5 Is a Novel Repressor of Lymphoid Enhancer Factor/T-cell Factor-driven Transcription*

Received for publication, May 31, 2005, and in revised form, October 11, 2005 Published, JBC Papers in Press, November 16, 2005, DOI 10.1074/jbc.M505869200

Stephen Mbigha Ghogomu, Stephanie van Venrooy, Martin Ritthaler, Doris Wedlich, and Dietmar Gradl[1]

From Zoologisches Institut II, Universität Karlsruhe (Technische Hochschule) , Kaiserstrasse 12, 76131 Karlsruhe, Germany

Activation of Wnt/β-catenin target genes is regulated by a heterodimer of β-catenin and the high mobility group box transcription factors of the lymphoid enhancer factor (LEF)/T-cell factor (TCF) family. In vertebrates, four LEF/TCF family members have been identified. They all contain a conserved β-catenin-binding motif at the N terminus and a highly conserved high mobility group box for DNA binding. The core sequence between these motifs is less conserved and contributes to the specific properties of the individual family members. To identify interacting proteins that allocate specific functions to the individual LEF/TCF transcription factors, we performed a yeast two-hybrid screen using the less conserved core sequence as bait. We isolated the murine LIM protein HIC-5 (hydrogen peroxide-induced clone 5; also termed ARA-55 (androgen receptor activator of 55 kDa)) and cloned the highly conserved *Xenopus* homolog. In addition, we report that the LIM domain-containing C-terminal half of HIC-5 binds to a conserved alternatively spliced exon in LEF/TCF transcription factors. Our functional analyses revealed that HIC-5 acts as negative regulator of a subset of LEF/TCF family members, which have been characterized as activators in reporter gene analyses and in the *Xenopus* axis induction assay. In addition, we observed a repressive interference of LEF/TCF family members with HIC-5-mediated activation of glucocorticoid-driven transcription, which again could be allocated to specific LEF/TCF subtypes. With the characterization of HIC-5 as a binding partner of the alternatively spliced exon in LEF/TCF transcription factors, we identified a novel molecular mechanism in the dialog of steroid and canonical Wnt signaling that is LEF/TCF subtype-dependent.

The four vertebrate lymphoid enhancer factor (LEF)[2]/T-cell factor (TCF) transcription factors TCF-1, TCF-3, TCF-4, and LEF-1 are the nuclear transducers of an activated Wnt/β-catenin pathway. They all contain a highly conserved β-catenin-binding domain and an even more conserved DNA-binding site, the high mobility group (HMG) box. In general, they are activated by recruiting the coactivator β-catenin, which is thought to replace the repressor Groucho (available at www.stanford.edu/~rnusse/wntwindow.html) (1, 2). Thereby, LEF/TCF target genes that are repressed in the absence of Wnt/β-catenin signaling become activated. Apart from a complex regulatory network in the cytoplasm that controls β-catenin stability and binding behavior, modulator proteins in the nucleus further decide the cell competence to respond to canonical Wnt signaling. In addition, the Wnt/β-catenin pathway is influenced by cross-talk with other signaling cascades, including transforming growth factor-β/SMAD (3), transforming growth factor-β/Nemo-like kinase (4), and Delta/Notch (5), and by protein modulators such as ALY (6) and PIAS (7).

Although the general view of LEF/TCF action is well understood, the functional specificity of individual LEF/TCF proteins are often ignored. Most of the LEF/TCF-binding proteins mentioned above do not discriminate between the different family members or splice variants. Furthermore, proteins such as CtBP and ALY that bind selectively to individual LEF/TCF proteins cannot completely explain their functional differences. For example, CtBP binds to PLSL(T/V) motifs in the C termini of TCF-3 and TCF-4E, resulting in repression of target genes (8, 9). But even after depletion of the CtBP-binding site, XTCF-3 still neither activates target genes nor induces a secondary axis in *Xenopus* embryos (10). Recently, we have shown that the core region between the β-catenin-binding domain and the HMG box confers specific properties to individual LEF/TCF transcriptions factors (10). This region has formerly been described as the interaction domain for Groucho (11, 12) and ALY (6). It contains a highly conserved alternatively spliced exon, which is termed exon IVa in TCF-1, exon VI in LEF-1, and exon VIII in TCF-4. Interestingly, a *Xenopus* LEF-1 RNA containing this exon has not been reported so far. Two small repressive peptide motifs adjacent to this exon are alternatively expressed in TCF-4 (10, 13). With the exception of this conserved exon, the sequence between the β-catenin-binding domain and the HMG box is less conserved and only poorly characterized.

In addition to the modulators of LEF/TCF activity mentioned above, steroid receptors not only bind β-catenin (14, 15), but also interact directly with TCF proteins (16–18). Steroid receptors belong to the family of ligand-activated zinc finger transcription factors and consist of an N-terminal transactivation domain, two zinc fingers, and a hormone-binding site. Upon ligand binding, the steroid receptor changes its conformation, which reduces the binding affinity of inhibitors (*e.g.* hsp90). After dimerization, it enters the nucleus and activates target genes. Interestingly, the cross-talk between Wnt/β-catenin signaling and steroid response is bidirectional: glucocorticoids inhibit the transcriptional activity of LEF/TCF proteins, whereas TCF proteins modulate estrogen receptor activity. Depending on individual TCF family members, the latter can result in enhancement (TCF-1) or suppression (TCF-4) of the estrogen-driven promoter response (16, 17).

Apart from the internal transactivation domain, several adaptor proteins that bind to steroid receptors and activate expression of target genes have been identified. Among them, ARA-55 (androgen receptor activator of 55 kDa; also named HIC-5 (hydrogen peroxide-induced clone 5)) was initially described as a component of the focal adhesion complex, in which it binds to the focal adhesion kinase (19, 20). HIC-5/ARA-5 (referred to below only as HIC-5) belongs to the paxillin family

* This work was supported by Deutsche Forschungsgemeinschaft Grant DG 1802. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY971603.

[1] To whom correspondence should be addressed. Tel.: 49-721-608-3988; Fax: 49-721-608-3992; E-mail: dietmar.gradl@zi2.uni-karlsruhe.de.

[2] The abbreviations used are: LEF, lymphoid enhancer factor; TCF, T-cell factor; HMG, high mobility group; PPARγ, peroxisome proliferator-activated receptor γ; X, *Xenopus*; m, murine; h, human; GST, glutathione *S*-transferase; MMTV, mouse mammary tumor virus; HEK, human embryonic kidney; CMV, cytomegalovirus.

of LIM proteins because it shares common protein-protein interaction motifs with paxillin: HIC-5 possesses three LD domains in the N-terminal half and four LIM domains in the C-terminal half.

Recent studies confirmed a nuclear role of HIC-5 as coactivator of steroid receptors (21, 22) and peroxisome proliferator-activated receptor γ (PPARγ) (23) and as co-regulator of transcription factors SMAD3 and Sp1 (24, 25). The pleiotropic functions of HIC-5 imply an important role as a regulatory protein, allowing the cell to integrate the input of different signaling cascades.

In this study, we identify HIC-5 as a novel binding partner of LEF/TCF proteins. The HIC-5 C terminus containing the LIM domains binds to a conserved exon in LEF/TCF proteins. This interaction of HIC-5 and LEF/TCF proteins is conserved in vertebrates and results in a complex that represses both LEF/TCF target gene activation and HIC-5-induced steroid receptor activation. However, this does not present a general regulatory mechanism of LEF/TCF target gene activation because only those family members that contain the conserved exon and that act as activators (*Xenopus* (X) TCF-4C, murine (m) LEF-1, human (h) TCF-1, and hTCF-4) are repressed by HIC-5. Other family members that activate target genes but do not contain the exon (*e.g.* XLEF-1) are not regulated by HIC-5. However, transcription factors that do not activate target gene promoters (TCF-3 and XTCF-4A) are not regulated by HIC-5. Instead, they repress HIC-5-induced steroid receptor activation. Thus, HIC-5 is a novel LEF/TCF binding partner that mediates the TCF subtype-specific cross-talk between Wnt/β-catenin signaling and steroid receptor activation.

EXPERIMENTAL PROCEDURES

Constructs—The coding regions of XLEF-1, XTCF-3, and XTCF-4 corresponding to amino acids 63–274, 63–328, and 63–353, respectively, were fused to the LexA DNA-binding domain in BTM116. Additionally, the same constructs and XTCF-3 amino acids 193–249 and XTCF-4 amino acids 220–316 were fused to glutathione *S*-transferase (GST) and His tags in pET-M30. hTCF-1, mTCF-3, and hTCF-4 in pcDNA3.1 were kindly provided by Hans Clevers, whereas hTCF-3 was from W. Birchmeier. psp64T3-mLEF-1 and pCS2-XLEF/XTCF constructs were as described previously (10). Full-length mHIC-5 constructs in pcDNA-3.1 and pGEX were kindly provided by Michael Stallcup. mHIC-5 fused to GST was separated in the N-terminal half containing the LD domains and in the C-terminal half containing the LIM domains by PCR.[3] The mouse mammary tumor virus (MMTV)-luciferase reporter construct was provided by Olivier Kassel. The *Xenopus fibronectin* reporter and the TOPFlash promoter have been described (26, 27).

Yeast Two-hybrid Screen—A mouse embryonic day 10 library cloned in the pVP16 vector was kindly provided by Jürgen Behrens. L40 yeast cells were transformed with BTM116 constructs and used for screening $\sim 1 \times 10^5$ transformants of library-transformed cells. After the interacting clones of the bait vector were cured, the clones were tested for specific interaction with XTCF-3 and XTCF-4 by mating with AMR70 previously transformed with the respective bait construct (28). Positive clones were isolated, sequenced, and analyzed.

Bacterial Expression of LEF/TCF Proteins—Transformed BL21(DE3) bacteria were induced at $A_{600} = 0.7$ with 1 mM isopropyl 1-thio-β-D-galactopyranoside for 4 h at 30 °C. After centrifugation, bacterial pellets were lysed in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.5) additionally containing 400 mM NaCl, 1 mg/ml lysozyme, and protease inhibitors. Aliquots of the cleared lysates were stored in liquid nitrogen.

Pull-down Assays—Bacterially expressed GST-tagged HIC-5 protein was immobilized on glutathione-Sepharose beads for 2 h at 4 °C in buffer A (10 mM Tris-Cl, pH 7.8, 150 mM NaCl, 1 mM $MgCl_2 \cdot 6H_2O$, 0.75 mM $CaCl_2 \cdot 2H_2O$, 2% Nonidet P-40, and protease inhibitors) and incubated with buffer A lysate from transfected human embryonic kidney (HEK) epithelial 293 cells. After binding for 2 h at 4 °C, the samples were washed three times with buffer A, boiled in SDS sample buffer for 5 min, and subjected to 10% SDS-PAGE. Proteins were transferred onto nitrocellulose, probed with anti-Myc monoclonal antibody 9E10, and revealed by the chemiluminescence reaction (ECL, Amersham Biosciences).

Immobilized GST-HIC-5 constructs and *in vitro* translated ^{35}S-labeled LEF/TCF proteins or immobilized GST-LEF/TCF constructs and *in vitro* translated ^{35}S-labeled HIC-5 proteins were incubated in buffer A, washed, eluted and separated on a 10% SDS gel. After Coomassie Blue staining, the gel was dried and subjected to PhosphorImager (Raytest) analysis to visualize the bound protein.

Injection Experiments—mRNA was synthesized *in vitro* using the mMESSAGE mMACHINE kit (Ambion, Inc.). 500 pg of LEF-1 or HIC-5 mRNA or 70 pg of XWNT-8 mRNA were injected into both ventral blastomeres of *Xenopus* four-cell stage embryos. Embryos were kept as described previously (29) and analyzed for the appearance of secondary axis, dorso-anteriorization, and target gene expression.

Transfection and Reporter Gene Assays—HeLa and HEK293 cells were routinely grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. HEK293 cells were transfected by calcium phosphate precipitation according to Gorman (30), and HeLa cells were transfected with MATra reagent (IBA GmbH, Göttingen, Germany) according to the manufacturer's recommendations. 48 h after transfection, cells were harvested. Reporter gene assays were performed as described (26). To analyze the glucocorticoid response, transfected cells were treated with 10 nM dexamethasone.

Reverse Transcription-PCR—1 μg of HeLa cell total RNA was reverse-transcribed using MMTV reverse transcriptase (Promega). cDNA corresponding to 20 ng of RNA was amplified for 28 (glyceraldehyde-3-phosphate dehydrogenase) or 34 (p21 and p27) cycles with the glyceraldehyde-3-phosphate dehydrogenase forward primer (5′-GTGGATATTGTTGCCATCAAT-3′) and reverse primer (5′-CGCTGTTGAAGTCAGAGGAG-3′), the p21 forward primer (5-ATGTCCGTCAGAACCCATG-3′) and reverse primer (5′-TTAGGGCTTCCTCTTGGAGA-3′, or the p27 forward primer (5′-GTCTAACGGGAGCCCTAGCC-3′) and reverse primer (5′-CTAACCCCGTCTGGCTGTCC-3′). 1 μg of *Xenopus* stage 10.5 total RNA was reverse-transcribed using MMTV reverse transcriptase. cDNA corresponding to 20 ng of RNA was amplified for 26 (histone H4) or 34 (*siamois* and *Xnr-3*) cycles with the histone H4 forward primer (5′-CGGGATAACATTCAGGGTATCACT-3′) and reverse primer (5′-ATCCATGGCGGTAACTGTCTTCCT-3), the *siamois* forward primer (5′-CTCCAGCCACCAGTACCAGAT-3′) and reverse primer (5′-GGGGAGAGTGGAAAGTGGTTG-3′, and the *Xnr-3* forward primer (5′-TCCACTTGTGCAGTTCCACAG-3′) and reverse primer (5′-ATCTCTTCATGGTGCCTCAGG-3′).

Isolation of Full-length XHIC-5—Using degenerated primers, we amplified a 500-bp fragment of the 5′-region of X*Hic-5* from stage 18 cDNA.[3] To obtain the entire open reading frame of X*Hic-5*, we screened a *Xenopus* tailbud λ-ZAP cDNA library with this fragment. The open reading frame of X*Hic-5* (GenBank™ accession number AY971603) was subcloned into the NcoI/XhoI sites of pCS2-Myc.

Immunostaining—HeLa cells were fixed with 3% formaldehyde and permeabilized by incubation for 8 min in phosphate-buffered saline

[3] Primer sequences are available upon request.

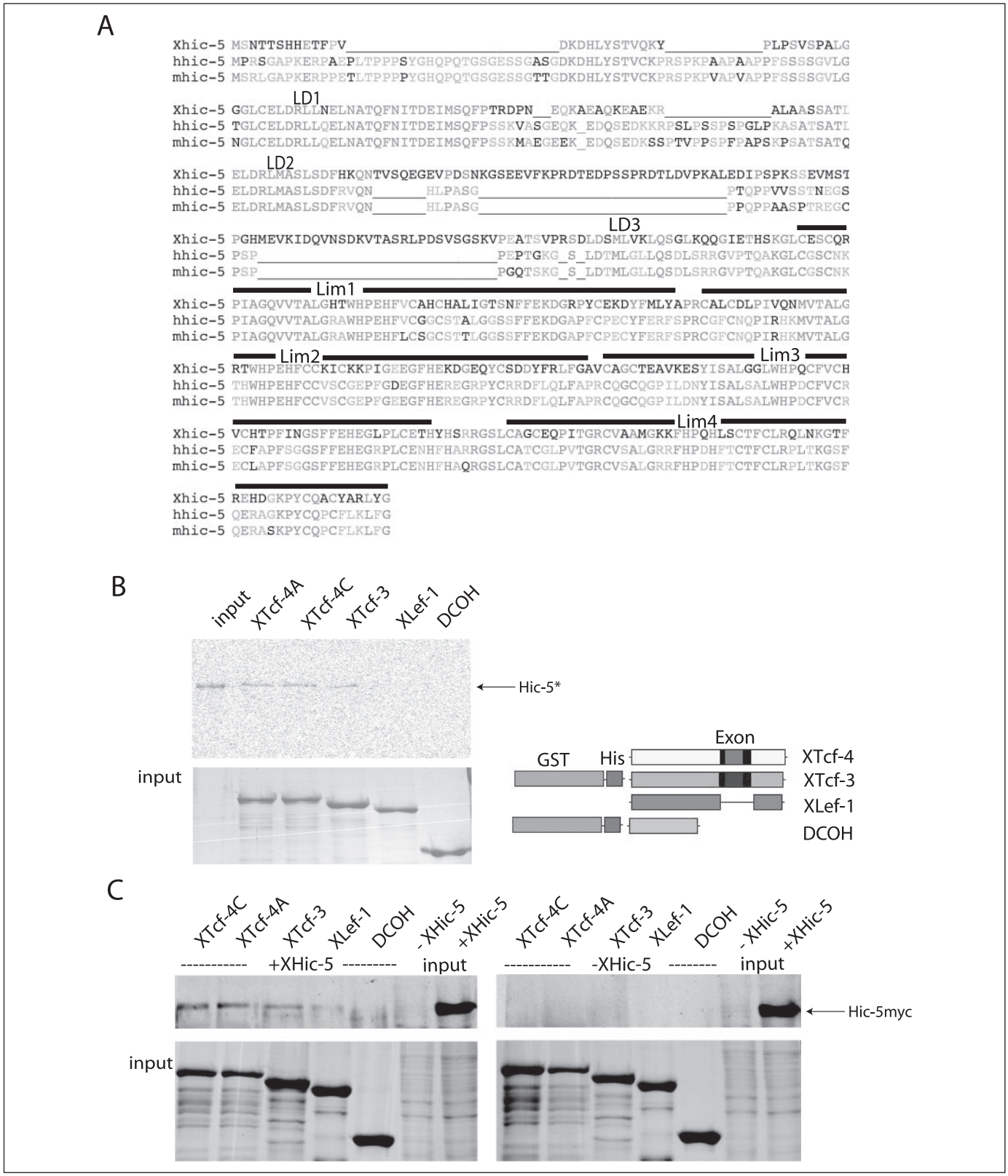

FIGURE 1. **XHIC-5 binds to *Xenopus* TCF proteins.** *A*, protein alignment of XHIC-5 with its human and murine orthologs reveals high homology in the LD and LIM domains. The three LD motifs are indicated, and the four LIM domains are illustrated by *black bars*. *B*, GST pull-down assay with *in vitro* translated XHIC-5 and immobilized *Xenopus* GST-LEF/TCF fusion proteins consisting of amino acids 63–274 for XLEF-1, amino acids 63–328 for XTCF-3, and amino acids 63–353 for XTCF-4 revealed that XHIC-5 specifically bound to XTCF-3 and XTCF-4, but only very weakly to XLEF-1. Recombinantly expressed GST-LEF/TCF fusion proteins were immobilized on glutathione-Sepharose and incubated with *in vitro* translated ^{35}S-labeled XHIC-5. *Upper panel*, autoradiograph; *lower panel*, same gel stained with Coomassie Blue to demonstrate equal loading. The GST-DCOH fusion protein served as a negative control. *C*, GST pull-down assays with transfected Myc-tagged XHIC-5 confirmed the interaction with XTCF-3 and XTCF-4. Again, wild-type XLEF-1 hardly precipitated with immobilized GST-LEF/TCF fusion proteins. Recombinantly expressed GST-LEF/TCF fusion proteins were immobilized on glutathione-Sepharose and incubated with crude cell extracts of HEK293 cells transiently transfected with Myc-tagged XHIC-5. *Upper panel*, immunoblot stained with Myc epitope-specific monoclonal antibody 9E10; *lower panel*, same gel stained with Coomassie Blue to demonstrate equal loading. The GST-DCOH fusion protein served as a negative control.

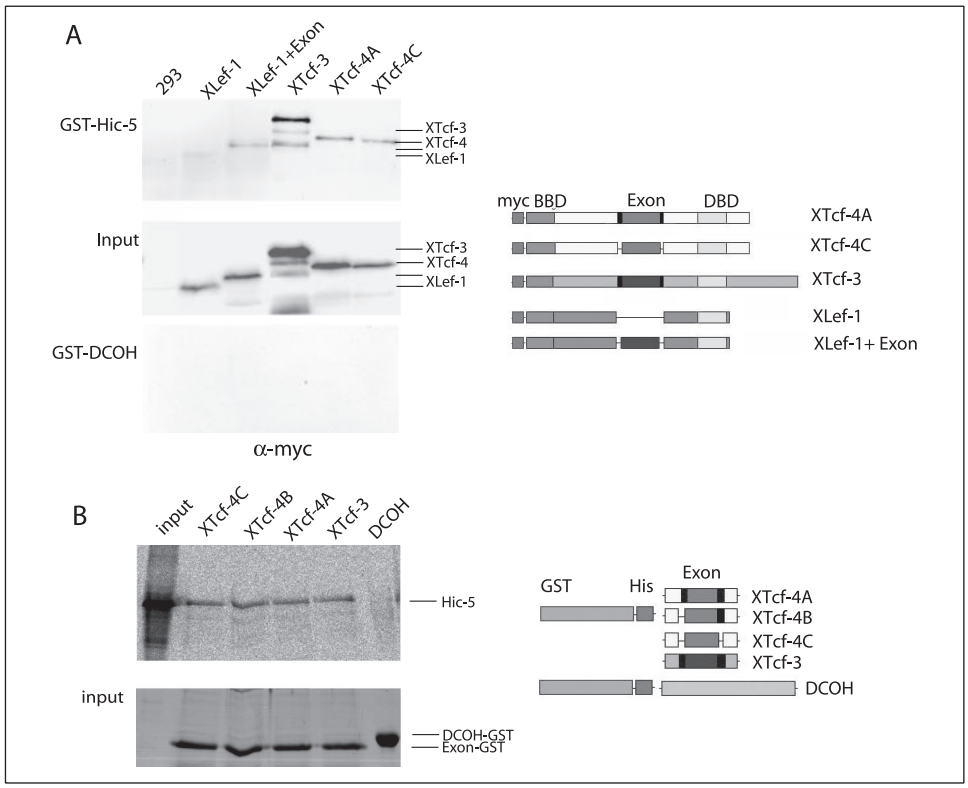

FIGURE 2. **The conserved exon in LEF/TCF is the binding domain for HIC-5.** *A*, recombinantly expressed GST-mHIC-5 was immobilized on glutathione-Sepharose and incubated with cell lysates from HEK293 cells transfected with the indicated Myc-tagged LEF/TCF constructs. Samples were analyzed by Western blotting with Myc epitope-specific monoclonal antibody 9E10 for the presence of the transfected constructs. GST-DCOH served as a negative control. *BBD*, β-catenin-binding domain; *DBD*, DNA-binding domain. *B*, recombinantly expressed exons of XTCF-3 and XTCF-4 were immobilized on glutathione-Sepharose and incubated with *in vitro* translated ^{35}S-labeled mHIC-5. *Upper panel*, autoradiograph; *lower panel*, same gel stained with Coomassie Blue to demonstrate equal loading.

containing 0.1% Triton X-100. Localization of the anti-HIC-5 (polyclonal; Santa Cruz Biotechnology, Inc.) and anti-TCF-3/4 and anti-LEF-1 (monoclonal; Pierce) primary antibodies was visualized with Cy2-conjugated goat anti-rabbit and Cy3-conjugated goat anti-mouse IgG, respectively.

RESULTS

XHIC-5 Discriminates between XTCF-3/4 and XLEF-1—To identify new binding partners, we screened a mouse embryonic day 10 library using the core domain (between the β-catenin-binding site and the HMG box) of *Xenopus* TCF-3 and TCF-4 as bait. We identified HIC-5 as a candidate protein that binds to both XTCF-3 and XTCF-4 (data not shown). The full-length X*Hic-5* cDNA (GenBank™ accession number AY971603) was isolated by screening a *Xenopus* tailbud λ-ZAP library. The XHIC-5 protein is 40% identical to its human ortholog. The similarity in the conserved three LD and four LIM domains ranges from 55% (LIM domain 2) to 100% (LD domain 2) (Fig. 1*A*). The most obvious differences between the *Xenopus* and mammalian HIC proteins are the absence of a 33-amino acid proline-rich region flanking a highly conserved part and the presence of a 55-amino acid acidic and serine-rich region between LD domains 2 and 3 in the *Xenopus* protein. To confirm the physical interaction found in the yeast two-hybrid screen, we carried out GST pull-down assays using bacterially expressed GST-LEF/TCF fusion proteins and *in vitro* translated ^{35}S-labeled XHIC-5. Indeed, we found that XHIC-5 bound to XTCF-3 and XTCF-4, but not to the GST control (Fig. 1*B*). We further confirmed the physical interaction using transfected Myc-tagged XHIC-5 and bacterially expressed GST-LEF/TCF fusion proteins (Fig. 1*C*). Interestingly, compared with XTCF-3 and XTCF-4, the binding of XHIC-5 to XLEF-1 was very weak (Fig. 1, *B* and *C*).

HIC-5 Binds to a Conserved Exon—The most obvious difference between XLEF-1 and XTCF-3/4 is a conserved exon that fails in XLEF-1. This exon corresponds to exon IVa in hTCF-1, exon VI in hLEF-1, and exon VIII in hTCF-4, all of which are known to be alternatively spliced. Alternative splicing of this exon has not been reported for the *Xenopus* homolog of LEF-1; instead, this exon is missing in all X*Lef-1* cDNAs reported so far (10, 31). By constructing chimeric proteins, we have recently shown that this exon promotes target gene activation (10). To confirm that this conserved exon is the HIC-5-binding domain, we transfected Myc-tagged *Xenopus* LEF/TCF constructs and analyzed their binding to recombinantly expressed HIC-5 in GST pull-down assays. Indeed, wild-type XLEF-1 hardly bound HIC-5, whereas a chimeric XLEF-1 construct containing the conserved exon of XTCF-3 (XLEF-1+Exon) was precipitated with immobilized GST-HIC-5 (Fig. 2*A*). Furthermore, *in vitro* translated HIC-5 bound to the recombinantly expressed exon of XTCF-3 and XTCF-4 (Fig. 2*B*). Interestingly, HIC-5 did not discriminate among XTCF-4A, -4B, and -4C, which differ in the presence of two small peptide motifs flanking the conserved exon (Fig. 2*B*).

The C Terminus (but Not the N Terminus) of HIC-5 Binds to LEF/TCF Proteins—We next tried to map the binding domain in HIC-5. As HIC-5 contains two putative protein-protein interaction sites, the LD and LIM domains, we fused the LD domain- and LIM domain-containing parts separately to GST. Therefore, we cut the protein into two halves, the N-terminal half containing the three LD domains and the C-terminal half containing the four LIM domains (Fig. 3). GST pull-down assays with *in vitro* translated LEF/TCF proteins revealed that the LEF/TCF-binding site is the LIM domain-containing C terminus, but not the LD domains (Fig. 3). Thus, the conserved exon of LEF/TCF proteins interacts with the C terminus, most likely with the LIM domains, of HIC-5. Because the binding of HIC-5 to LEF/TCF proteins was observed for the *Xenopus* and murine proteins and also between the corresponding binding partners of different species, we conclude that the interaction is conserved in vertebrates.

HIC-5 Suppresses LEF/TCF-induced Target Gene Activation—Next, we asked whether the physical interaction between LEF/TCF proteins

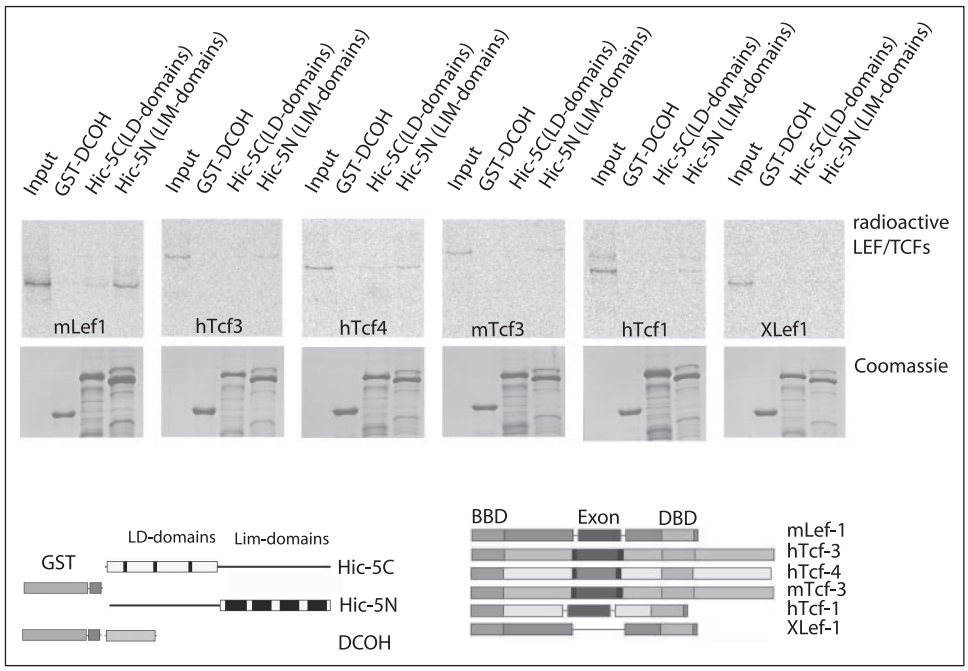

FIGURE 3. **LEF/TCF proteins bind to the LIM domain-containing C-terminal half of HIC-5.** The recombinantly expressed C-terminal half (GST-HIC-5ΔN, LIM domains, amino acids 212–430) and N-terminal half (GST-HIC-5ΔC, LD domains, amino acids 1–202) of mHIC-5 were immobilized on glutathione-Sepharose and incubated with *in vitro* translated ^{35}S-labeled murine and human LEF/TCF proteins. *Upper panels*, autoradiographs; *lower panels*, same gels stained with Coomassie Blue to demonstrate equal loading. GST-DCOH served as a negative control. *BBD*, β-catenin-binding domain; *DBD*, DNA-binding domain.

and HIC-5 results in activation or repression of Wnt/β-catenin target genes. Therefore, we cotransfected HEK293 cells with LEF/TCF reporter constructs, HIC-5, and different LEF/TCF expression constructs. Transfected HIC-5 (both the murine and *Xenopus* orthologs) had only a minor effect on the activity of the TOPFlash promoter by itself. Consistent with previously published data (10, 13), XTCF-4C and XLEF-1 (but not XTCF-4A and XTCF-3) activated the TOPFlash promoter in HEK293 cells (Fig. 4*A*). In the presence of HIC-5, however, XTCF-4C did not activate the TOPFlash promoter, and activation by the chimeric XLEF-1+Exon construct was drastically reduced. In the case of XTCF-4C, promoter activation dropped from 2.1 to 1.2-fold (mHIC-5) or 0.8-fold (XHIC-5) and, in the case of XLEF-1+Exon, from 4.1- to 1.9- or 1.5-fold, respectively (Fig. 4*A*). Consistent with the observation that HIC-5 bound to a conserved exon present in XTCF-3, XTCF-4, and XLEF-1+Exon but missing in XLEF-1, we found no effect of HIC-5 on TOPFlash activation via XLEF-1. The promoter was activated by 1.5–2-fold irrespective of whether HIC-5 was cotransfected or not (Fig. 4*A*). The specificity of TOPFlash activation and repression is documented by cotransfection of the control promoter FOPFlash, which was neither activated by XLEF-1+Exon nor repressed by HIC-5 (Fig. 4*B*).

This repressive function of HIC-5 is conserved among vertebrates because the results were similar and even more pronounced when we studied mHIC-5 in combination with human or murine LEF/TCF proteins. mLEF-1, hTCF-1, and h-TCF-4 activated the TOPFlash promoter by 3.1–3.5-fold. As shown for the *Xenopus* LEF/TCF proteins, cotransfection of HIC-5 inhibited mammalian LEF/TCF-induced promoter activation (Fig. 4*C*). Although TCF-3 contains the conserved exon, it did not activate the TOPFlash promoter and was not regulated by HIC-5.

The regulation of Wnt target genes by HIC-5 is not restricted to the artificial TOPFlash promoter. We observed a similar response when we used the *Xenopus* fibronectin promoter (Fig. 4*D*). Again, cotransfection of HIC-5 prevented promoter activation by XLEF-1+Exon (1.7-fold *versus* 3.2-fold) and XTCF-4C (1.6-fold *versus* 2.3-fold), but not by XLEF-1. Thus, the binding of the conserved exon of LEF/TCF proteins to the C-terminal half of HIC-5 is conserved among vertebrates and prevents activation of Wnt/β-catenin target gene promoters.

HIC-5 Suppresses Secondary Axis Formation—Ectopic activation of the Wnt/β-catenin cascade in the ventral hemisphere of *Xenopus* embryos results in the induction of a secondary Spemann organizer and subsequently in the appearance of a secondary body axis. The only LEF/TCF family member that mimics an activated Wnt/β-catenin cascade and induces a secondary body axis upon ventral injection is LEF-1. We showed recently that the frequency of secondary axis formation is higher following mLEF-1 injection than following XLEF-1 injection and that this difference is due to the presence of the conserved exon (10). If HIC-5 is indeed a general repressor that binds to the conserved exon, it should suppress mLEF-1 (but not XLEF-1)-induced secondary axis formation in *Xenopus* embryos. Therefore, we co-injected 500 pg of HIC-5 mRNA together with 500 pg of mLEF-1 or XLEF mRNA into both ventral blastomeres of *Xenopus* four-cell stage embryos and scored the appearance of a secondary axis. As expected, co-injected HIC-5 reduced the frequency of secondary axis formation induced by mLEF-1 from 33 to 15%, but had no effect on the frequency of XLEF-1-induced secondary axis formation (Fig. 5, *A* and *B*).

After injection of 70 pg of XWNT-8 mRNA, most of the embryos (94%, $n = 80$) showed the most severe canonical Wnt phenotype, a complete dorso-anteriorization. This phenotype is best seen by a ring-shaped cement gland (Fig. 5*C*). After co-injection of HIC-5, only 3% ($n = 104$) of the injected embryos showed this complete dorso-anteriorization. Instead, 58% of the embryos now showed a partial rescue as seen by the appearance of a secondary axis, and 39% revealed a complete rescue as seen by the appearance of a single axis (Fig. 5*C*).

Consistent with the suppression of canonical Wnt signaling in reporter gene and axis induction assays, co-injected HIC-5 counteracted the XWNT-8-induced *siamois* and *Xnr-3* induction (Fig. 5*D*). Thus, HIC-5 represses canonical Wnt signaling *in vivo*.

LEF/TCF Proteins Repress Steroid Receptor Target Genes in a HIC-5-dependent Manner—We next investigated whether the physical interaction of HIC-5 and LEF/TCF proteins also affects the activity of other HIC-5-regulated target gene promoters. Because HIC-5 is known to be a coactivator of steroid receptors (21, 22, 32), we analyzed whether LEF/TCF proteins influence the function of HIC-5 as coactivator of the glucocorticoid receptor by reporter gene assays in HeLa cells. HeLa cells

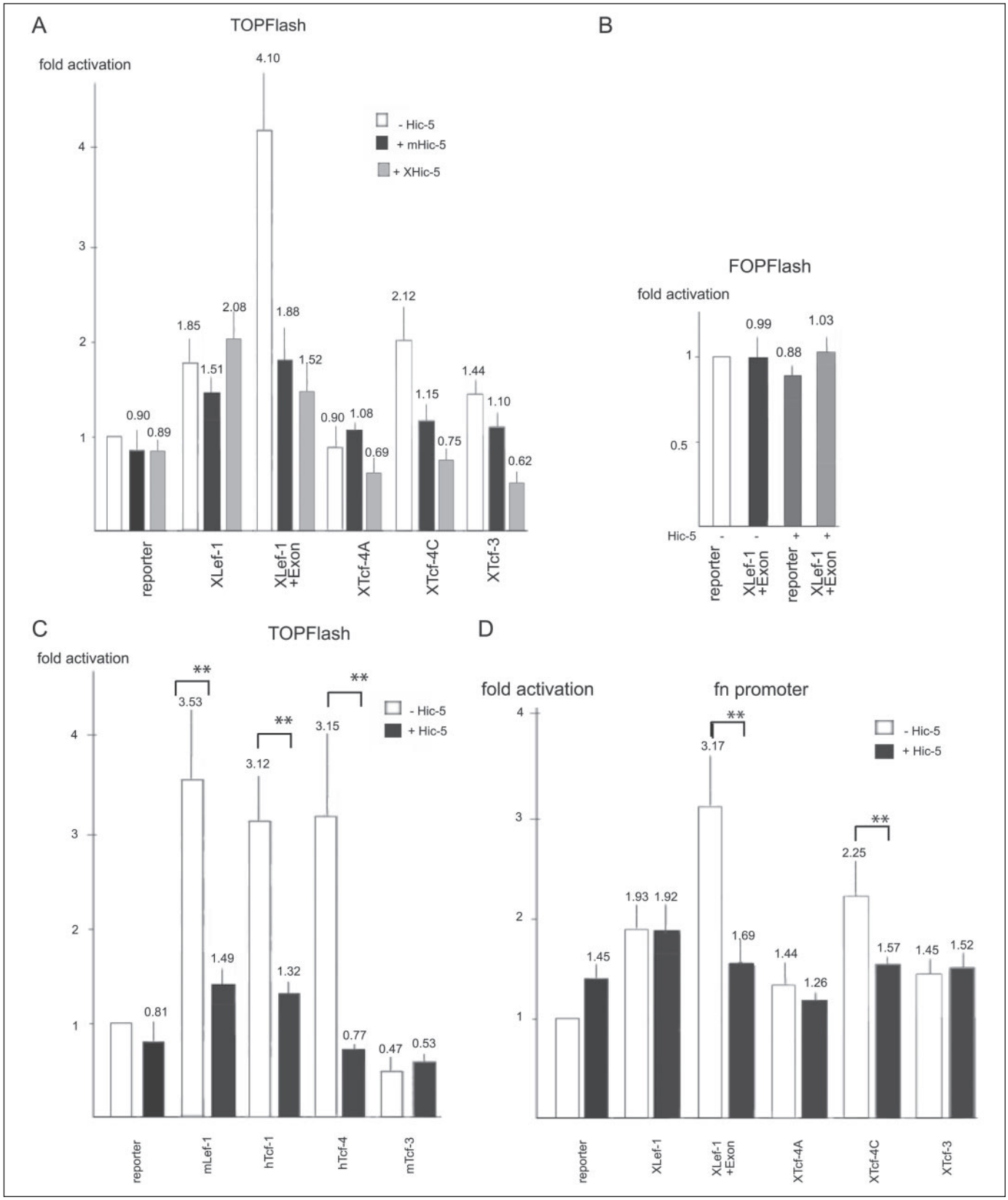

FIGURE 4. **HIC-5 represses LEF/TCF-induced target gene promoter activation.** *A*, HEK293 cells were cotransfected with TOPFlash, cytomegalovirus (CMV)-β-galactosidase for normalization, and the indicated LEF/TCF constructs in the absence (*white bars*) or presence of mHIC-5 (*black bars*) or XHIC-5 (*gray bars*). *B*, HIC-5 did not regulate the FOPFlash promoter in either the presence or absence of XLEF-1+Exon. HEK293 cells were cotransfected with FOPFlash, CMV-β-galactosidase for normalization, and XLEF-1+Exon in the presence or absence of mHIC-5. *C*, HEK293 cells were cotransfected with TOPFlash, CMV-β-galactosidase, and the indicated human and murine LEF/TCF proteins in the absence (*white bars*) or presence (*black bars*) of mHIC-5. *D*, HEK293 cells were cotransfected with the *Xenopus* fibronectin (*fn*) promoter, CMV-β-galactosidase, and the indicated LEF/TCF proteins in the absence (*white bars*) or presence (*black bars*) of mHIC-5. Each *bar* represents the average of 6–14 transfections. The *error bars* indicate the S.E. **, significant difference ($p < 0.05$, Student's *t* test).

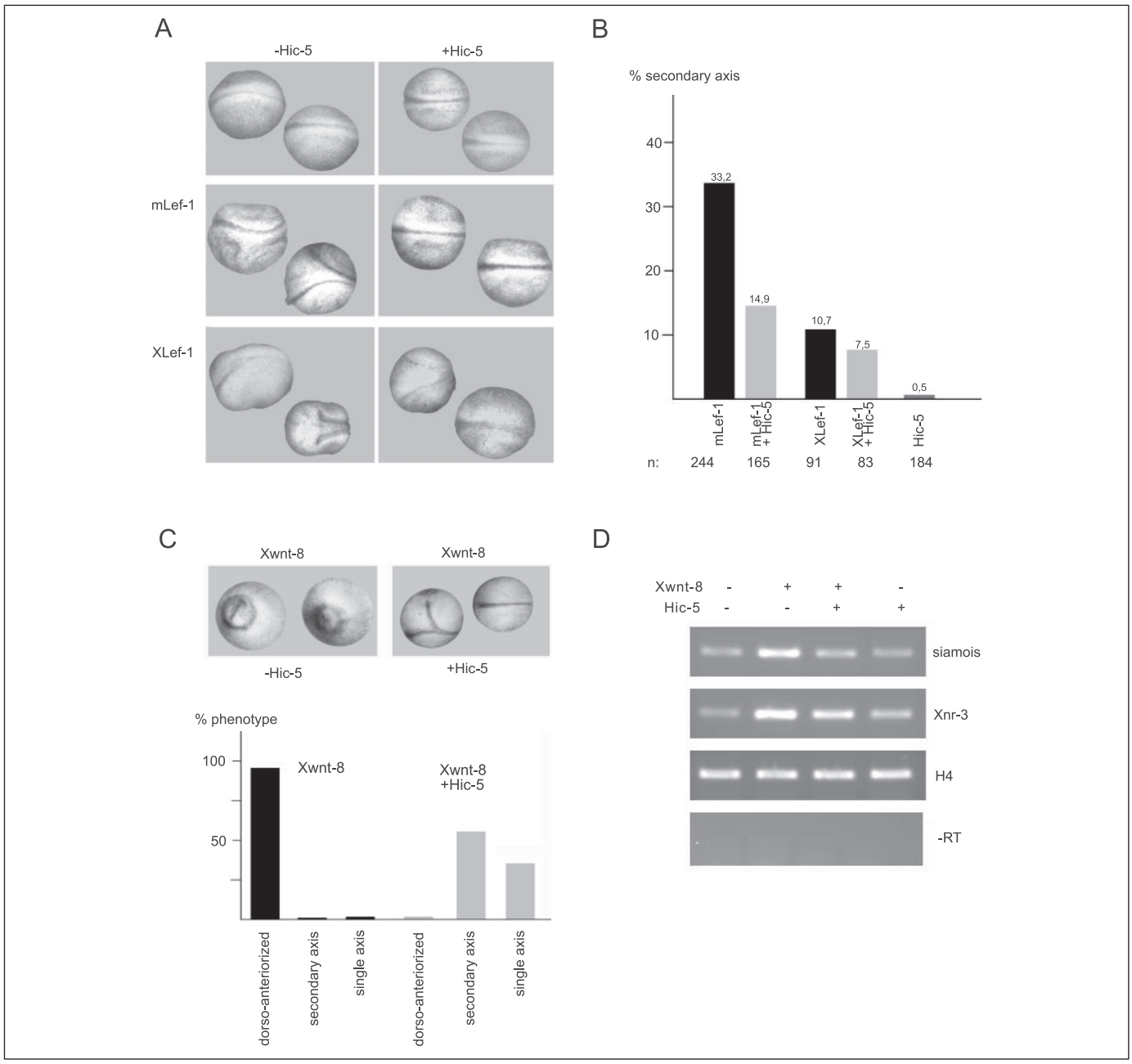

FIGURE 5. **HIC-5 suppresses canonical Wnt signaling in *Xenopus* embryos.** Both ventral blastomeres of *Xenopus* four-cell stage embryos were injected with 500 pg or mLEF-1, XLEF-1, or mHIC-5 mRNA or co-injected with 500 pg of mHIC-5 mRNA + 500 pg of mLEF-1 or XLEF-1 mRNA. Embryos were cultivated until the neurula stage and analyzed for secondary axis formation. *A*, the phenotypes of injected embryos are shown. Secondary axes induced by mLEF-1 and XLEF-1 are best seen by the appearance of a secondary neural tube. *B*, secondary axis formation was quantified. *n*, number of injected embryos. *C*, embryos injected with 70 pg of XWNT-8 RNA revealed complete dorso-anteriorization, which is best seen by the appearance of a ring-shaped cement gland. Co-injection of 500 pg of HIC-5 mRNA resulted in a partial rescue and the appearance of secondary axis or in a complete rescue and the appearance of a single axis. *D*, expression of the Wnt target genes *siamois* and *Xnr-3* was analyzed by reverse transcription-PCR in injected and uninjected stage 10.5 *Xenopus* embryos. *H4*, amplification of the histone H4 housekeeping gene; *−RT*, control amplification without reverse transcription.

endogenously express HIC-5 and contain substantial amounts of HIC-5 protein in the nucleus, where it co-localizes with LEF/TCF proteins (Fig. 6*A*). The MMTV long terminal repeat is a model promoter for studying steroid receptor activation because it directs target gene regulation by glucocorticoid, mineralocorticoid, progesterone, and androgen receptors.

Dexamethasone activated the MMTV promoter in a dose-dependent manner (Fig. 6*B*). Up to 1 nM dexamethasone activated the promoter by ~3-fold and 10 nM dexamethasone by ~9-fold, and further increases to 20 nM resulted in 15-fold activation. We also observed this dose dependence at the level of target gene transcription. Expression of $p21^{cip1}$ and $p27^{kip1}$ was almost undetectable at 1 nM dexamethasone, but robustly increased at higher concentrations (Fig. 6*C*).

Cotransfection of HIC-5 at moderate dexamethasone concentrations (10 nM) enhanced promoter activation from ~9–20-fold (Fig. 6, *D* and *E*). Overexpression of XLEF-1 and XLEF-1+Exon did not influence the glucocorticoid response by itself; but in the presence of the conserved exon, activation mediated by cotransfected HIC-5 was decreased (Fig. 6*D*). XTCF-3 and XTCF-4 reduced the dexamethasone-induced activation and, more strikingly, turned coexpressed HIC-5 into a repressor. Thus, LEF/TCF proteins that contain the conserved exon repress HIC-5-mediated glucocorticoid receptor activation.

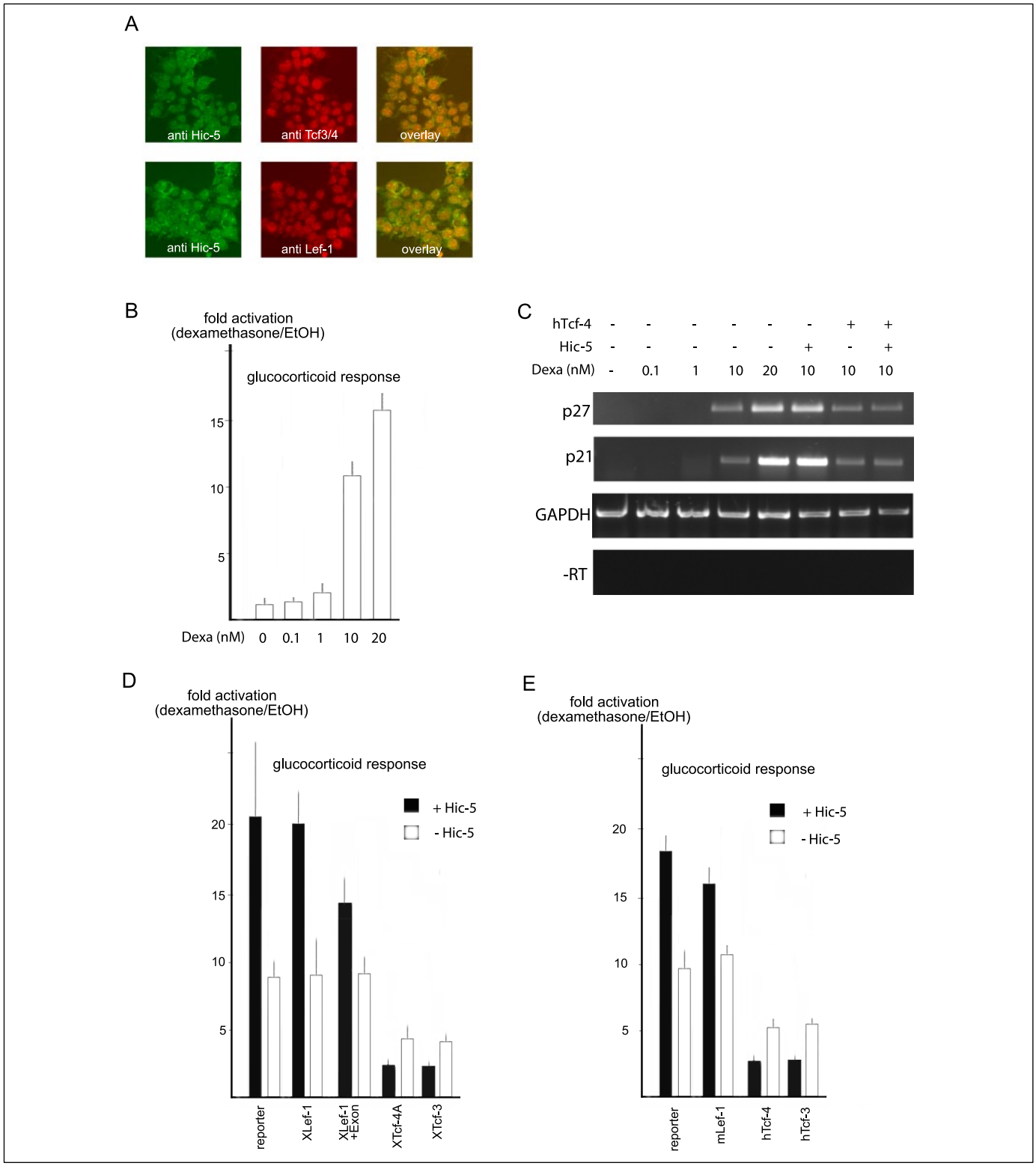

FIGURE 6. **LEF/TCF proteins suppress HIC-5-induced steroid receptor activation.** *A*, shown are the results from double staining of HeLa cells with a polyclonal antibody against HIC-5 (*green*) and a monoclonal antibody against TCF-3/4 or LEF-1 (*red*). Overlay of the HIC-5 signal with LEF/TCF signals revealed that HeLa cells contain substantial amounts of HIC-5 protein in the nucleus, where it co-localizes with LEF/TCF proteins. *B*, HeLa cells were cotransfected with MMTV-luciferase and CMV-β-galactosidase for normalization and treated with the indicated amounts of dexamethasone (*Dexa*). Each *bar* represents the mean value of at least seven transfections. The *error bars* indicate the S.E. *C*, reverse transcription-PCR analysis revealed that expression of the glucocorticoid receptor target genes $p21^{cip1}$ and $p27^{kip1}$ depends on the hormone concentration and the levels of HIC-5 and TCF. *GAPDH*, amplification of the housekeeping gene; *−RT*, control amplification of the housekeeping gene without reverse transcription. *D*, HeLa cells were cotransfected with MMTV-luciferase and CMV-β-galactosidase for normalization and the indicated *Xenopus* LEF/TCF constructs in the presence (*black bars*) or absence (*white bars*) of mHIC-5. The glucocorticoid receptor was activated by adding 10 nM dexamethasone dissolved in ethanol or by adding the solvent alone. Shown is the -fold activation by dexamethasone treatment normalized to ethanol treatment. *E*, the conditions were the same as described for *D*, but with mammalian LEF/TCF proteins. Each *bar* represents the mean value of at least seven transfections. The *error bars* indicate the S.E.

We obtained similar results when we cotransfected the human androgen receptor and studied the response to dihydrotestosterone (data not shown) or when we used mammalian LEF/TCF proteins instead of the *Xenopus* homologs (Fig. 6*E*). Again, the promoter was not regulated by LEF-1, but LEF-1 slightly repressed activation via cotransfected HIC-5. TCF-3 and TCF-4 repressed the dexamethasone-induced activation and, like their *Xenopus* homologs, turned coexpressed HIC-5 into a repressor.

We found that expression of the glucocorticoid receptor target genes $p21^{cip1}$ and $p27^{kip1}$ was induced by HIC-5, but not by TCF-4, and that TCF-4 suppressed the HIC-5-induced activation (Fig. 6*C*), consistent with the reporter gene data. Thus, our data indicate that the physical interaction of HIC-5 and LEF/TCF proteins results in a repressive complex. We identified HIC-5 as a conserved mediator that regulates the cross-talk between LEF/TCF proteins and steroid receptors in a LEF/TCF subtype-specific manner.

DISCUSSION

In this study, we have identified HIC-5 as a novel LEF/TCF binding partner. The binding of the LIM domain-containing C terminus of HIC-5 to LEF/TCF transcription factors results in a repressive complex that prevents LEF/TCF-induced target gene activation and axis induction. Up to now, only a few LEF/TCF-interacting proteins such as SMAD4 (3) and ALY (33) that promote target gene activation have been identified. All others, including Groucho (2) and CtBP (9), act as corepressors. Thus, the identification of HIC-5 as a new corepressor of LEF/TCF proteins further supports the idea that repression by a LEF/TCF·corepressor complex is the primary regulatory principle for Wnt/β-catenin target genes.

Because the interaction between LEF/TCF proteins and HIC-5 was found to be conserved in different species, we suggest that HIC-5 is a general important regulator of canonical Wnt signaling in vertebrates. We mapped a conserved and alternatively spliced exon as the binding domain in LEF/TCF proteins. Notable, unlike Groucho, HIC-5 did not completely block LEF/TCF-driven target gene activation. Instead, HIC-5 regulated only a subset of LEF/TCF proteins. Thus, we identified a molecular mechanism through which LEF/TCF subtypes acquire individual properties.

The conserved LIM domains of HIC-5 are known to bind a multitude of proteins as well as DNA (23, 24, 34, 35). In this study, we have demonstrated for the first time that the LIM domain-containing C terminus also binds to LEF/TCF transcription factors. The binding domain in LEF/TCF proteins overlaps with the Groucho-binding domain, which has been shown to be the region between the β-catenin-binding site and the HMG box (12, 36). Nevertheless, the binding domains for Groucho and HIC-5 are not identical because XLEF-1 binds HIC-5 only weakly (this study), but still binds Groucho (13). We identified a highly conserved alternatively spliced exon as the interaction domain in LEF/TCF proteins, and this exon has 80% identity between LEF-1 and TCF-4 and 70% identity between TCF-3 and TCF-4. In *Xenopus*, this exon is present in the mRNA of XTCF-3 and XTCF-4, but not XLEF-1. The interaction of HIC-5 with the alternatively spliced exon is conserved in vertebrates because we found it in different vertebrate species and even across species. Interestingly, this exon is part of context-dependent activation domain B in hLEF-1 (37) and thus distinct from the ALY interaction site, which is located predominantly in context-dependent activation domain A (33). Using chimeric proteins, we recently identified this exon as an activating element (10). Although the mode of activation via the conserved exon is still unknown, the binding of the corepressor HIC-5 might provide an explanation for its absence in XLEF-1: lack of this exon prevents inappropriate repression by HIC-5. For different LEF/TCF family members, it has been shown that the HIC-5 interaction site is alternatively spliced (38, 39), indicating that the regulation of Wnt/β-catenin signaling by HIC-5 is restricted to distinct LEF/TCF isoforms. Thus, in addition to alternatively expressed C termini, which define whether the general repressor CtBP can bind to TCF-3 and TCF-4 or not (8, 9), alternative splicing in the core domain between the β-catenin-binding site and the HMG box modulates the activity of LEF/TCF transcription factors by recruiting HIC-5.

Although HIC-5 has been characterized as coactivator for Sp1 and steroid receptors, we have demonstrated here that the interaction with LEF/TCF proteins results in a repressive complex. This repression was not restricted to the regulation of the artificial LEF/TCF-responsive promoter TOPFlash or the promoter of the Wnt/β-catenin target gene fibronectin, but was also observed *in vivo* in injected *Xenopus* embryos.

The pleiotropic functions of HIC-5 on several transcriptional regulators, including steroid receptors (21, 22), PPARγ (23), Sp1 (24), and SMAD (25), imply that it might be a threshold protein that controls the communication of different signaling cascades. It has recently been shown that, as an interaction partner of PPARγ, HIC-5 induces the epithelial program in the intestine (23). In contrast, TCF-4 has been shown to be essential for the maintenance of a stem cell population in the intestine (40) and for maturation of Paneth cells in intestinal crypts (41), and inappropriate Wnt signaling in the colon results in cancer formation (42). Although PPARγ is expressed in microvilli and TCF-4 expression is restricted to crypt cells, HIC-5 expression increases from the crypt to the microvillus tip (23, 41). One might speculate that the interaction of HIC-5 with PPARγ (23) and LEF/TCF proteins (this study) is involved in the decision of whether the intestinal cells continue to proliferate (high TCF-4 and low HIC-5 and PPARγ) or whether they start to differentiate to epithelial cells (low TCF-4 and high HIC-5 and PPARγ).

The cross-talk between steroid receptor signaling and canonical Wnt signaling is regulated at several levels. We have provided evidence that, in addition to the direct interaction of steroid receptors with β-catenin (15) and LEF/TCF proteins (16, 17) a TCF·HIC-5 complex represses the glucocorticoid response. Interestingly, the different LEF/TCF proteins regulated the glucocorticoid response in different ways. Although the HIC-5-mediated glucocorticoid response was not altered by LEF-1 without the exon, it was repressed by the exon-containing chimeric protein and exon-containing mLEF-1. This repression was even more pronounced by cotransfected TCF-3 or TCF-4. Thus, HIC-5 mediates a LEF/TCF subtype-specific regulation of the glucocorticoid response, with LEF-1·HIC-5 acting as a weak repressor and TCF-3·HIC-5 and TCF-4·HIC-5 acting as robust repressors both of glucocorticoid-responsive promoters and of expression of the endogenous glucocorticoid receptor target genes $p21^{cip1}$ and $p27^{kip1}$. Taken together, our data reveal that HIC-5 is a new LEF/TCF subtype-specific corepressor and that the interaction of the LIM domain-containing C-terminal half of HIC-5 with an alternatively spliced exon in LEF/TCF proteins defines a new level of the cross-talk between Wnt/β-catenin signaling and steroid receptor activation.

Acknowledgments—We thank J. Behrens, W. Birchmeier, A. Cato, H. Clevers, O. Kassel, and M. Stallcup for providing plasmids and constructs. We are grateful to A. Tomsche for technical assistance.

REFERENCES

1. Logan, C. Y., and Nusse, R. (2004) *Annu. Rev. Cell Dev. Biol.* **20,** 781–810
2. Daniels, D. L., and Weis, W. I. (2005) *Nat. Struct. Mol. Biol.* **4,** 364–371

3. Nishita, M., Hashimoto, M. K., Ogata, S., Laurent, M. N., Ueno, N., Shibuya, H., and Cho, K. W. (2000) *Nature* **403,** 781–785
4. Ishitani, T., Ninomiya-Tsuji, J., and Matsumoto, K. (2003) *Mol. Cell. Biol.* **23,** 1379–1389
5. Galceran, J., Sustmann, C., Hsu, S.-C., Folberth, S., and Grosschedl, R. (2004) *Genes Dev.* **18,** 2718–2723
6. Hsu, S.-C., Galceran, J., and Grosschedl, R. (1998) *Mol. Cell. Biol.* **18,** 4807–4818
7. Sachdev, S., Bruhn, L., Sieber, H., Pichler, A., Melchior, F., and Grosschedl, R. (2001) *Genes Dev.* **15,** 3088–3103
8. Valenta, T., Lukas, J., and Korinek, V. (2003) *Nucleic Acids Res.* **31,** 2369–2380
9. Brannon, M., Brown, J. D., Bates, R., Kimelman, D., and Moon, R. T. (1999) *Development* (*Camb.*) **126,** 3159–3170
10. Gradl, D., König, A., and Wedlich, D. (2002) *J. Biol. Chem.* **277,** 14159–14171
11. Levanon, D., Goldstein, R. E., Bernstein, Y., Tang, H., Goldenberg, D., Stifani, S., Paroush, Z., and Groner, Y. (1998) *Proc. Nat. Acad. Sci. U. S. A.* **95,** 11590–11595
12. Brantjes, H., Roose, J., van de Werering, M., and Clevers, H. (2001) *Nucleic Acids Res.* **29,** 1410–1419
13. Pukrop, T., Gradl, D., Henningfeld, K., Knöchel, W., Wedlich, D., and Kühl, M. (2001) *J. Biol. Chem.* **276,** 8968–8978
14. Yang, F., Li, X., Sharma, M., Sasaki, C. Y., Longo, D. L., Lim, B., and Sun, Z. (2002) *J. Biol. Chem.* **277,** 11336–11344
15. Song, L.-N., Herrell, R., Byers, S., Shah, S., Wilson, E. M., and Gelmann, E. P. (2003) *Mol. Cell. Biol.* **23,** 1674–1687
16. Amir, A. L., Barua, M., McKnight, N. C., Cheng, S., Yuan, X., and Balk, S. P. (2003) *J. Biol. Chem.* **278,** 30828–30834
17. El-Tanani, M., Fernig, D. G., Barraclough, R., Green, C., and Rudland, P. (2001) *J. Biol. Chem.* **276,** 41675–41682
18. Smith, E., and Frenkel, B. (2005) *J. Biol. Chem.* **280,** 2388–2394
19. Fujita, H., Kamiguchi, K., Cho, D., Shibanuma, M., Morimoto, C., and Tachibana, K. (1998) *J. Biol. Chem.* **273,** 26516–26521
20. Nishiya, N., Tachibana, K., Shibanuma, M., Mashimo, J. I., and Nose, K. (2001) *Mol. Cell. Biol.* **21,** 5332–5345
21. Yang, L., Guerrero, J., Hong, H., DeFranco, D. B., Stallcup, M. R. (2000) *Mol. Biol. Cell* **11,** 2007–2018
22. Guerrero-Santoro, J., Yang, L., Stallcup, M. R., and DeFranco, D. B. (2004) *J. Cell. Biochem.* **92,** 810–819
23. Drori, S., Girnun, G. D., Tou, L., Szwaya, J. D., Mueller, E., Kia, X., Shivdasani, R. A., and Spiegelman, B. M. (2005) *Genes Dev.* **19,** 362–375
24. Shibanuma, M., Kim-Kaneyama, J.-I., Sato, R., and Nose, K. (2004) *J. Cell. Biochem.* **91,** 633–645
25. Wang, H., Song, K., Sponseller, T. L., and Danielpour, D. (2005) *J. Biol. Chem.* **280,** 5154–5162
26. Gradl, D., Kühl, M., and Wedlich, D. (1999) *Mol. Cell. Biol.* **19,** 5576–5587
27. Korinek, V., Barker, N., Morin, P. J., van Wichen, D., de Weger, R., Kinzler, K. W., Vogelstein, B., and Clevers, B. (1997) *Science* **275,** 1784–1787
28. Hollenberg, S. M., Sternglanz, R., Cheng, P. F., and Weintraub, H. (1995) *Mol. Cell. Biol.* **7,** 3813–3822
29. Kühl, M., Sheldahl, L. C., Malbon, C. C., and Moon, R. T. (2000) *J. Biol. Chem.* **275,** 12701–12711
30. Gorman, C. (1985) in *DNA Cloning: a Practical Approach* (Glover, D. M., ed) pp. 143–190, IRL Press, Oxford
31. Molenaar, M., van de Wetering, M., Oosterwegel, M., Peterson-Maduro, J., Godsave, S., Korinek, V., Roose, J., Destree, O., and Clevers, H. (1996) *Cell* **86,** 391–399
32. Miyoshi, Y., Ishiguro, H., Uemura, H., Fujinami, K., Miyamoto, H., Miyoshi, Y., Kitamura, H., and Kubota, Y. (2003) *Prostate* **56,** 280–286
33. Bruhn, L., Munnerlyn, A., and Grosschedl, R. (1997) *Genes Dev.* **11,** 640–653
34. Nishiya, N., Sabe, H., Nose, K., and Shibanuma, M. (1998) *Nucleic Acids Res.* **26,** 4267–4273
35. Jia, Y., Ransom, R. F., Shibanuma, M., Liu, C., Welsh, M. J., and Smoyer, W. E. (2001) *J. Biol. Chem.* **276,** 39911–39918
36. Roose, J., Molenaar, M., Peterson, J., Hurenkamp, J., Brantjes, H., Moerer, P., van de Wetering, M., Destree, O., and Clevers, H. (1998) *Nature* **395,** 608–612
37. Carlsson, P., Waterman, M. L., and Jones, K. A. (1993) *Genes Dev.* **7,** 2418–2430
38. van de Wetering, M., Castrop, J., Korinek, V., and Clevers, H. (1996) *Mol. Cell. Biol.* **16,** 745–752
39. Hovanes, K., Li, T. W. H., and Waterman, L. (2000) *Nucleic Acids Res.* **28,** 1994–2003
40. Korinek, V., Barker, N., Moerer, P., van Donselaar, E., Huls, G., Peters, P. J., and Clevers, H. (1998) *Nat. Genet.* **19,** 379–383
41. Van Es, J. H., Jay, P., Gregorieff, A., van Gijin, M. E., Jonkheer, S., Hatzis, P., Thiele, A., van dern Born, M., Begthel, H., Brabletz, T., Taketo, M. M., and Clevers, H. (2005) *Nat. Cell Biol.* **7,** 381–386
42. Peifer, M. (1997) *Science* **275,** 1752–1753