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HMG box transcription factor TCF-4's interaction with CtBP1 controls the expression of the Wnt target Axin2/Conductin in human embryonic kidney cells

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ABSTRACT

Members of the Tcf/Lef family of the HMG box transcription factors are nuclear effectors of the Wnt signal transduction pathway. Upon Wnt signaling, TCF/LEF proteins interact with β-catenin and activate transcription of target genes, while, in the absence of the Wnt signal, TCFs function as transcriptional repressors. All vertebrate Tcf/Lef transcription factors associate with TLE/Groucho-related co-repressors, and here we provide evidence for an interaction between the C-terminus of the TCF-4 HMG box protein and the C-terminal binding protein 1 (CtBP1) transcriptional co-repressor. Using Wnt-1-stimulated human embryonic kidney 293 cells, we show that CtBP1 represses the transcriptional activity of a Tcf/β-catenin-dependent synthetic promoter and, furthermore, decreases the expression of the endogenous Wnt target, Axin2/Conductin. The CtBP1-mediated repression was alleviated by trichostatin A treatment, indicating that the CtBP inhibitory mechanism is dependent on the activity of histone deacetylases.

INTRODUCTION

Members of the Wnt family of growth factors control numerous developmental processes and aberrant activation of Wnt genes, and Wnt signaling is also implicated in deregulated cell growth and cancer (reviewed in 1,2). Insights into the mechanisms of the Wnt signaling pathway have emerged from genetic studies in *Drosophila*, biochemical experiments in cell culture, ectopic gene expression in early *Xenopus* embryos and from target gene inactivation in mice (3). A striking characteristic of Wnt signaling is its conservation during evolution. Components of the Wnt signaling pathway have been found in such evolutionary distant species as slime mould *Dictyostelium*, diploblastic metazoan Hydra, *Caenorhabditis elegans* and in higher vertebrates, including mammals (4–6). This indicates that the Wnt signaling cascade is one of the primary regulatory mechanisms controlling cell fate decisions in multicellular organisms.

A brief outline of the canonical Wnt pathway is the following (for more detailed information, refer to the Wnt homepage at http://www.stanford.edu/~rnusse/wntwindow.html). The interaction of extracellular Wnt ligands with the Frizzled/LRP receptor complex results in increased intracellular levels of β-catenin in the target cell (7–9). In unstimulated cells, free cytoplasmic β-catenin is marked on N-terminal serine and threonine residues by phosphorylation, and then ubiquitinated and rapidly degraded by the proteasome pathway (10–13). The β-catenin degradation machinery includes casein kinase Iα (CKIα) and a multiprotein cytoplasmic complex containing serine–threonine kinase GSK-3, Axin1 (or its homolog Axin2/Conductin) and the APC tumor suppressor (14–20). When cells are stimulated by Wnt proteins, the cytoplasmic phosphoprotein Dishevelled is recruited to the plasma membrane and, by a so far unknown mechanism, inhibits the function of the GSK-3/Axin/APC complex (21,22). The unphosporylated stable β-catenin molecules accumulate in the cytoplasm and also translocate into the nucleus. Nuclear β-catenin forms heterocomplexes with TCF/LEF proteins (23–25). The TCF/β-catenin heterodimers act as bipartite transcription factors and activate the expression of the specific Wnt responsive genes (26,27). Approximately 50 Wnt targets have been identified to date, and several of these genes encode proteins related to cell cycle regulation, e.g. c-myc, Cyclin D1 and Pitx2 (28–31).

Relay and final processing of the Wnt signal in the target cell is controlled at several levels. In the extracellular space, proteins of the Dkk, WIF and SFRP families interact directly with Wnt ligands or their receptors and inhibit transmission of the signal into the cell (32–37). In the cytoplasm, LIT-1/Nemo-like kinase phosphorylates TCFs and regulates the DNA binding and subcellular distribution of TCF/LEF factors (38–40). β-Catenin activity in the nucleus may be promoted by interaction with a number of proteins including Pontin52, Brg-1, Pygopus, p300 and Lines (41–45). β-Catenin-mediated transcription is repressed by Reptin52 (a Pontin52 homolog), ICAT, XSox17α/β and XSox-3 protein (46,47). Other interacting partners modulate the function of TCF/LEF proteins.

In mammals, the Tcf/Lef family consists of four genes: *Tcf-1*, *Lef-1*, *Tcf-3* and *Tcf-4*. All TCF/LEF proteins display several common structural features (48,49). They contain a nearly identical DNA-binding domain, the HMG box, recognizing the consensus sequence A/T A/T CAAA. The

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extreme N-terminus harbors the β-catenin interacting domain. Although capable of binding to DNA, TCF/LEF proteins possess only limited ability to activate transcription. They might rather be viewed as nuclear vehicles targeting other auxiliary proteins to a specific set of promoters. β-Catenin possesses a strong transcription activation domain, so it is an example of an activating interacting partner. In contrast, all mammalian TCFs also associate with TLE/Groucho co-repressors and, in the absence of the Wnt signal, repress the transcription of the Tcf-dependent synthetic reporter or endogenous Tcf targets (50). TLE/Groucho proteins interact with the hypoacetylated N-terminal tail of histone H3 and also with human histone deacetylase 1 (HDAC 1) and its *Drosophila* homolog Rpd3 (51,52). This suggests that the function of TLE/Groucho is to form a specialized repressive chromatin structure that prevents the inappropriate activation of β-catenin/TCF target genes.

The participation of Wnt signaling in multiple developmental programs leads to the question of how β-catenin/TCF complexes discriminate between different subsets of all potential Wnt target genes at a given time and cellular background. Functional differences between individual TCF/LEF proteins could be one possible explanation. Although *Tcf/Lef* mRNAs are expressed in complex and often overlapping patterns during embryogenesis and in adult tissues, gene targeting experiments in mice revealed that Tcf-1, Lef-1 and Tcf-4 execute different developmental programs and their functions are only partially redundant (53–55). Additionally, several observations indicated that TCFs interact with distinct partners and show individual context-dependent DNA-binding and transactivation properties. For example, it has been reported that LEF-1 cooperates with microphthalmia-associated transcription factor (MITF) to up-regulate the dopachrome tautomerase gene promoter in melanocytes (56), and that activation of the T-cell receptor alpha enhancer by LEF-1 depends on ALY, a nuclear protein that specifically associates with LEF-1 and AML-1 (57). The activity of LEF-1 is suppressed by its association with PIASy, a nuclear matrix-associated SUMO E3 ligase, which sequesters LEF-1 into nuclear bodies (58) and, furthermore, direct interaction of LEF-1 with HDAC 1 mediates a repressive effect of LEF-1 in the absence of Wnt signaling (59). Another Tcf family member, TCF-3, interacts with casein kinase 1ε (CK1ε), and phosphorylation of TCF-3 by CK1ε stimulates its binding to β-catenin (60). In *Xenopus laevis*, XTCF-3 requires XCtBP to function as a transcriptional repressor (61). Recently, Hecht and Stemmler showed that TCF-4 and LEF-1 exhibit significant differences in regulating the promoter of two Wnt-responsive genes, *Cdx-1* and *Siamois*, in human embryonic kidney cells (62). Cdx-1 activation strictly depended on the promoter-specific transactivation domain located at the TCF-4 C-terminus. In addition, multiple isoforms are generated from *Tcf/Lef* mRNAs by way of alternative splicing or by use of dual promoters, and these isoforms display diverse functional properties (63–65). Thus, selective interaction of members of the Tcf/Lef family with various partners and their intrinsic DNA-binding properties can support the execution of different developmental programs.

To study the regulatory mechanisms of Wnt signaling, we screened for proteins interacting with human TCF-4. In this report, we show that the C-terminal binding protein 1 (CtBP1) associates with the TCF-4 C-terminus and that CtBP1 overexpression in Wnt-1-stimulated 293 cells represses transcription from the Wnt-responsive reporter and also decreases the activity of the endogenous Axin2 promoter. The CtBP1-mediated repression is alleviated by trichostatin A treatment, indicating that the CtBP inhibitory mechanism is dependent on the action of histone deacetylases.

MATERIALS AND METHODS

Plasmids

Constructs encoding proteins containing N-terminal EGFP were generated in pEGFP/C vector (BD Clontech). Myc-tagged cDNAs were prepared in pK-Myc vector. This vector was created by replacing the EGFP region in pEGFP/C with a T7 promoter and Myc tag coding sequence. Full-length cDNAs without N-terminal tags contained either natural initiator or a PCR-introduced consensus Kozak sequence. The expression plasmids for Tcf-4 were derived from human *Tcf-4E* cDNA (GenBank accession No. NM_030756) using naturally occurring restriction enzyme sites: *Sma*I (position 236 bp from ATG), *Nsi*I (995) and *Nde*I (1159). Plasmids encoding full-length or truncated forms of mCtBP1 were prepared in an analogous way using *Bgl*II, *Pvu*II, *Kpn*I and *Pst*I internal restriction enzyme sites. Detailed information about the constructs is available upon request. The mouse *Lef-1* cDNA was obtained from a mouse testis cDNA library (a gift from Stoil Dimitrov, IMG AS CR, Prague, Czech Republic). The TCF4–LEF-1 chimeric construct was generated by an exchange of the 3′ coding region in *Tcf-4* with the C-terminal coding sequence of mouse *Lef-1* using the *Apa*LI restriction enzyme site conserved in both genes. Human *β-catenin* cDNA was obtained from a bone marrow MATCHMAKER cDNA library (BD Clontech). Human *CtBP1* cDNA was amplified from the cDNA of 293 cells, mouse *CtBP2* was cloned from day-19 mouse embryo cDNA. Mouse *Wnt-1* cDNA was a gift from Marc van Dijk (University Hospital Utrecht, Utrecht, The Netherlands). For retroviral transduction, *Wnt-1* or EGFP-tagged *mCtBP1* were ligated into pLNIT (generously provided by Fred H. Gage, The Salk Institute, La Jolla, CA) or pLNCX vector (BD Clontech). Platinum Pfx DNA Polymerase (Invitrogen) was used for all PCR amplifications. PCR-derived constructs were verified by sequencing.

Yeast two-hybrid screen

A pre-transformed mouse 17-day embryo MATCHMAKER cDNA library (Clontech) was screened with the last 185 amino acids of the human TCF-4 protein fused to the GAL-4 DNA-binding domain (DBD) in pGBKT7 as bait. Positive clones were subjected to the specificity test using the GAL-4 DBD or GAL-4 DBD–lamin fusion protein as bait. Those clones that interacted specifically with the GAL-4 DBD–TCF-4 bait were sequenced.

Cell culture, transfections and retrovirus infection

Human embryonic kidney (HEK) cell lines 293, monkey kidney cells COS-7, rat fibroblast cell line Rat2, EcoPack-293 and AmphoPack-293 (Clontech) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented

with 10% fetal bovine serum (Hyclone). Transfections were performed using the lipofectamine reagent (Invitrogen), as described by the manufacturer. For retrovirus production, ecotropic EcoPack-293 or amphotropic AmphoPack-293 packaging cells were seeded in 6-well plates 12 h prior to transfection and then transfected with 3 μg of a particular retroviral expression construct. After 24 h, the growth medium was replaced with 2 ml of fresh medium. After a further 24 h incubation, the supernatant containing non-replicating forms of the virus was harvested. Target cell lines were infected in 6-well plates with the virus supernatant in the presence of 5 μg of polybrene (Sigma) per ml. Twenty-four hours later, the medium was replaced by complete DMEM supplemented with either G418 (concentration 1 mg per ml) or hygromycin B (0.5 mg per ml; both antibiotics were purchased from Invitrogen) and doxycycline (Sigma), concentration 1 μg per ml [expression of the transduced cDNAs in pLNIT is regulated by doxycycline (66)]. After a 10 day selection, the growing (resistant) cells were washed extensively with DMEM and subsequently cultivated in fresh selective medium without doxycycline. The expression of the transferred genes was confirmed by western blot analysis.

Reporter gene assays, trichostatin A treatment and Wnt-1 stimulation experiments

For reporter gene assays, 293 cells were seeded into 24-well plates (approximately 10^5 cells per well) and transfected 2 h later with a lipofectamine mixture containing 50 ng *Renilla* pRL-SV40 plasmid (Promega) as an internal control, 400 ng luciferase reporter plasmid and up to 1 μg of the particular expression vector. The total amount of DNA was kept constant by adding empty expression vector where necessary. The firefly luciferase reporter constructs pTOPFLASH and pFOPFLASH, containing either three copies of the optimal Tcf motif GATCAAAGG or three copies of the mutant motif GGCCAAAGG, respectively, were described previously (27). The lipofectamine–DNA mixtures for reporter gene assays in 293-EGFP-CtBP1/Dox contained 50 ng *Renilla* pRL-SV40 internal control plasmid and 400 ng luciferase reporter plasmid only, i.e. no empty vector was added. COS-7 cells were seeded in 12-well plates and transfected by lipofection with 50 ng *Renilla* pRL-SV40 plasmid, 400 ng luciferase reporter plasmid and various combinations of the following plasmids (see Fig. 5A): TCF-4 expression vector (400 ng), β-catenin expression vector (800 ng) and CtBP1 expression vector (0.5–2 μg). Additional empty pK-Myc plasmid was added when necessary to make the total amount of DNA equivalent. Firefly and *Renilla* luciferase activities in cell lysates were determined 15 h post-transfection using the dual luciferase system (Promega), according to the protocol supplied by the manufacturer, and a single tube luminometer Sirius (Berthold). All reporter gene assays were done in triplicate. The reporter gene activities shown are average values, along with the standard deviations from at least three independent experiments after normalization against the *Renilla* luciferase activities. Treatments with the deacetylase inhibitor trichostatin A (Sigma) were performed using the drug at 300 nM concentration for 12 h. Wnt-1 stimulation experiments were performed as follows. 3×10^5 293 cells were seeded into 6-well plates and transfected by lipofectamine–DNA mixtures. The amounts of individual DNAs in the mixtures were proportionally increased by a factor of three as compared to the experiments in 24-well plates. Four hours post-transfection, the cells were washed extensively with DMEM and then 1×10^5 Rat2-Wnt-1/Const cells expressing stable levels of Wnt-1 protein were plated over the target cells. Parental Rat2 cells were used as a negative control. After co-cultivation for 3–30 h, the cells were harvested together and further processed for the reporter gene assay, RNA isolation, electrophoretic-mobility shift assay (EMSA) or immunoblotting. When feeders with inducible *Wnt-1* expression (Rat2-Wnt-1/Dox) were used, the experimental procedure was modified as follows. We regularly grew the feeder cells in a complete medium supplemented with 2 ng of doxycycline per ml. This low concentration of the antibiotic was sufficient to suppress Wnt-1 production, and, additionally, Wnt-1 protein started to appear almost instantly upon the removal of doxycycline. Transfected 293 cells were covered with Rat2-Wnt-1/Dox cells and co-cultivated in medium without doxycycline. Cell cultures growing in the presence of doxycycline (5 ng per ml) were used as negative controls. The regulation of CtBP1 expression in 293-EGFP–CtBP/Dox cells was rather 'leaky', thus we cultivated these cells in higher concentrations of Dox (1 μg/ml). The full induction of the CtBP1 transgene was then achieved during 15 h growth in the absence of doxycycline.

GST interaction assays

Full-length GST–mCtBP1 and GST–mCtBP2 fusion proteins were expressed in the BL21 (DE3) strain of *Escherichia coli* using the pET-42b vector (Novagen). Full-length or truncated TCF-4 and full-length mCtBP1 protein were produced *in vitro* using the TNT Coupled Reticulocyte System (Promega) and corresponding pK-Myc constructs. All coupled transcription–translations were performed in a total volume of 50 μl using 10 μl of [^{35}S]methionine (ICN Biomedicals) per reaction. Twenty microliters of radiolabeled TCFs or mCtBP1 were incubated with GST–mCtBP1 or GST–mCtBP2 proteins bound to glutathione–Sepharose 4B beads (Amersham Pharmacia Biotech) in GST binding buffer [phosphate-buffered saline, pH 7.4, 0.5% (v/v) Nonidet P-40, 20 μM NADH (Sigma) and Protease Inhibitor Cocktail (Sigma)] for 1 h at 4°C. Beads were collected by centrifugation and washed five times in GST binding buffer. Bound proteins were separated by SDS–PAGE and analyzed by autoradiography.

Antibodies and immunoblotting

The following commercially available antibodies were used: β-catenin, CtBP, mouse monoclonal (Santa Cruz); EGFP, mouse monoclonal (BD Clontech); Myc tag, mouse monoclonal 9E10 (Roche Molecular Biochemicals); TCF-4, mouse monoclonal (Sigma); Wnt-1, rabbit polyclonal (Santa Cruz); and LEF-1, goat polyclonal (Santa Cruz). To monitor protein expression, 2×10^5 293 cells, seeded into 6-well plates, were transfected with 3 μg of expression vector. Twenty-four hours later cells were harvested and disrupted directly in SDS–PAGE sample buffer. The cell lysates were cleared of cell debris and chromosomal DNA by ultracentrifugation at 200 000 *g*, then loaded onto SDS–PAGE gels, transferred to polyvinylidene fluoride membranes (Pall Gelman Laboratory)

and immunoblotted with the appropriate antibodies. Proteins were visualized with an enhanced chemiluminiscence system (Pierce).

EMSA

The nuclear extracts were prepared according to Dignam *et al*. (67), snap frozen in liquid nitrogen and stored at –70°C. The final buffer composition was 20 mM HEPES, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 20% glycerol. Extracts were prepared from intact nuclei that were washed three times to avoid contamination with cytoplasmic β-catenin. As the optimal Tcf/Lef probe, we used a double-stranded 19-nucleotide oligomer AGAACCCTTTGATCTT-AGG; the control probe was AGAACCCTTTGGCCTTAGG. The oligonucleotides were end-labeled using T4 polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP (3000 Ci/mmol; ICN Biomedicals). A typical binding reaction contained 8 µg of nuclear protein, 1 ng of radiolabeled probe and 300 ng of poly-(deoxyinosic–deoxycytidylic) acid (poly [dIdC]; Sigma) in 25 µl of binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, 10 mM HEPES, pH 6.9 and 10% glycerol). Samples were incubated for 30 min at room temperature, an antibody was added and the samples were incubated for a further 30 min. The binding reactions were loaded onto 5% polyacrylamide gels with 0.5× Tris–borate–EDTA running buffer. Electrophoresis was carried out at 15°C at 220 V constant voltage. Gels were transferred onto Whatman paper, dried and exposed to the BAS-phosphoimager screen (Fuji).

Real-time quantitative RT–PCR

One hundred micrograms of total RNA from Wnt-1-stimulated 293 or control cells isolated by the guanidine thiocyanate (Fluka) method (68) were further purified using the Micro-to-Midi total RNA isolation system (Invitrogen). Random or oligo dT-primed cDNA was prepared in a 20 µl reaction from 2.5 µg of total RNA using Superscript II RNAseH$^-$ reverse transcriptase (Invitrogen). One percent of the resulting cDNA was used for one quantitative PCR reaction. The 10 µl reaction mixtures also contained 1× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), acetylated BSA (final concentration 3 µg per ml; New England Biolabs), Sybr Green I (final dilution 1:40 000; Amresco) and 5 pmol of each specific primer. All primers were calculated using Primer 3 computer services at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi/primer3_www.cgi. The primer pairs were designed and tested to be specific for human genes, i.e. the primers did not prime on rat or mouse cDNA. We used primers for the following human genes (the primers are written in the 5′ to 3′ direction, the first primer is derived from the plus and the second primer from the minus DNA strand): *Axin1*, CCTGTGGTCTACCCGTGTCT, GCTATGAGGAGTGGTCCAGG; *Axin2*, CTGGCTTTGGT-GAACTGTTG, AGTTGCTCACAGCCAAGACA; *GAPDH*, CACCACACTGAATCTCCCCT, CCCCTCTTCAAGGGG-TCTAC; *CyclinD1*, CCATCCAGTGGAGGTTTGTC, AGC-GTATCGTAGGAGTGGGA; *SDHA*, AGATTGGCACCT-AGTGGCTG, ACAAAGGTAAGTGCCACGCT; *CtBP1*, CCTTCGCGTTCCTCGTTA, AAGAACGTTCATGGGAG-AATAA; *Tcf-1*, CCTCTGCCTCCCTAGCTTTT, ATGGGG-GAGATGGGTAGAGA; *Lef-1*, CTGCTAGAGACGCT-GATCCA, TGGCTCTTGCAGTAGACGAA. cDNAs were produced from at least two independent RNA isolations, and the PCR reactions were performed in triplicate for each primer set. The specificity of the PCR products was verified by sequencing. Two housekeeping genes, *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *Succinate dehydrogenase complex, subunit A* (*SDHA*), were used as internal control genes to standardize the quality of different cDNA preparations (69). The cycling was performed in a LightCycler instrument (Roche Molecular Biochemicals). The results were analyzed using the LightCycler 3 software package. The relative abundance of *Axin1* and *Axin2* mRNA in 293 cells stimulated by Rat-2-Wnt-1/Const cells versus control 293 cells (co-cultivated with the parental Rat2 cell line) was derived from the average CT values of each triplicate after normalizing against the levels of *SDHA* cDNA. Results of a representative experiment are shown.

RESULTS

TCF-4 interacts with the transcriptional co-repressors CtBP1 and CtBP2

To gain insight into the molecular function of TCF-4 protein, we started a search for novel TCF-4-interacting proteins using a yeast two-hybrid system. We selected a 180 amino acid region distal to the HMG box covering the C-terminus of TCF-4 as bait. This region has a unique primary structure within the Tcf/Lef family and displays only limited homology to the C-terminus of TCF-3, the closest relative of TCF-4 (Fig. 1). One strong positive obtained from a day-17 mouse embryo cDNA library corresponded to *CtBP1*, a widely expressed transcriptional co-repressor. The cDNA encoded the entire 430 amino acid CtBP1 polypeptide. The CtBP1–Gal4 activation domain fusion protein interacted specifically with the TCF-4 bait and not with the Gal-4 DBD alone or with the Gal-4 DBD–lamin fusion protein, respectively (see Materials and Methods).

We further attempted to delineate the minimal region of CtBP1 required for association with TCF-4. We generated a series of N- and C-terminal deletions of CtBP1 fused to the Gal4 activating domain and tested their interaction with Gal4 DBD–TCF-4 in a yeast two-hybrid system. Each truncation of CtBP1 displayed a dramatically reduced ability to bind TCF-4 (data not shown). This indicates the complex multidomain mode of interaction between CtBP1 and TCF-4.

Human CtBP1 was originally discovered during a screen for cellular proteins that complex with the C-terminal region of the adenoviral E1A protein (70). Subsequently, a highly homologous human polypeptide termed CtBP2 was identified by analysis of EST database sequences (71). Other vertebrates such as rodents and *Xenopus* also contain two *CtBP* homologs, while invertebrates have a single *CtBP* gene. The CtBP proteins bind to a short sequence motif PLDLS conserved among the E1A proteins of all human and primate adenoviruses. Different variants of this motif are also present in other CtBP-interacting partners that function mainly as negative regulators of transcription (reviewed in 72). Two putative CtBP-binding sequences are also present in the C-termini of TCF-3 and TCF-4 proteins (Fig. 1); Brannon and others showed that simultaneous mutations in both these sites abolished the association of XTCF-3 with XCtBP, a *Xenopus* homolog of CtBP1, *in vitro* (61).

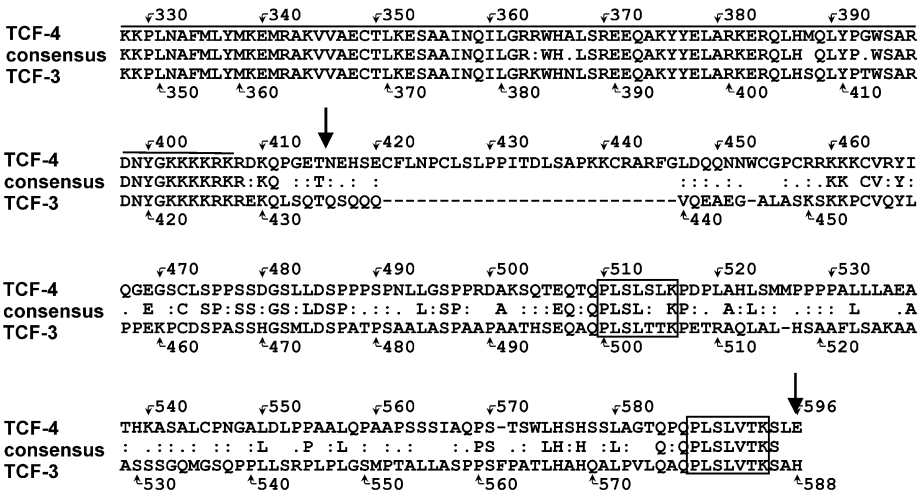

Figure 1. Amino acid comparison of the C-terminal regions of human TCF-4 and TCF-3. Amino acid identities are indicated by corresponding letters; amino acid similarities are indicated by double dots (closely related residues) or single dots (distantly related residues). Sequences were aligned with CLUSTALW. Overlining indicates highly conserved HMG box sequences; two putative CtBP binding sites are boxed. The arrows depict the amino acid sequence used in a yeast two-hybrid screen.

Given the putative CtBP-binding motifs in the C-terminus of TCF-4, we tested the interaction using bacterially purified GST–CtBP1 or GST–CtBP2 and various forms of TCF-4 translated *in vitro* (Fig. 2A). As shown in Figure 2B, a GST fusion protein containing complete CTBP1 interacted with [^{35}S]methionine-labeled full-length TCF-4 (FLTCF-4) or with its C-terminal fragment (ΔN_{Nde}TCF-4). Truncated proteins that lacked either sequences distal to the HMG box (TCF-4–LEF-1 chimera) or the entire C-terminal region (ΔC_{Nsi}TCF-4) failed to interact with mCtBP1. GST-tagged CtBP2 showed an identical mode of interaction (not shown), and, additionally, we observed efficient heterodimerization of CtBP1 and CtBP2 *in vitro* (Fig. 2B). Taken together, these data indicate that TCF-4 associates with CtBP1 and CtBP2 proteins and that the TCF-4 C-terminus is indispensable for the interaction.

Wnt signaling is activated in 293 cells co-cultivated with Wnt-1 protein-producing feeder cells

By retrovirus infection we generated several polyclonal rat fibroblast cell lines with constitutive (Rat2-Wnt-1/Const) or doxycycline-repressible (Rat2-Wnt-1/Dox) expression of the mouse *Wnt-1* gene (Fig. 3A). Because Wnt molecules possess only limited solubility and mostly stay attached to the cell surface of the producing cell, we activated Wnt signaling in 293 cells by co-cultivation with *Wnt-1*-expressing feeder cells. The human embryonic kidney 293 cells selected for the study activate the Tcf/β-catenin-dependent reporter if transiently transfected with *Wnt-1* expression plasmid, and, additionally, express high levels of *Tcf-4* mRNA and produce predominantly a longer variant, i.e. the TCF-4E form, of TCF-4 protein, which contains both CtBP-binding motifs [(27); Fig. 3B].

As shown in Figure 3C, Wnt-1-producing feeders stimulated the Tcf-reporter pTOPFLASH 6–7-fold as compared to control feeders. Co-transfection of an expression construct encoding dominant negative TCF-4, which lacks the N-terminal β-catenin interacting domain (ΔN_{Sma}TCF-4), completely abrogated the reporter gene activity, whereas a β-catenin expression plasmid strongly enhanced transcription from the pTOPFLASH reporter. The activity of the negative control reporter pFOPFLASH did not display any significant changes. We also employed a gel retardation supershift assay to determine whether Wnt signaling induces a nuclear TCF-4/β-catenin complex in stimulated 293 cells. We did not detect TCF–DNA complexes in nuclear extracts prepared from parental Rat2 fibroblast cells or Rat2-Wnt-1-transfected cells (not shown), therefore we performed gel retardations with compound nuclear extracts isolated from 293 and feeder cells. Using an optimal Tcf-motif as probe, we readily obtained two types of TCF–DNA complexes from parental and from both unstimulated and stimulated 293 cells; one of these complexes could be supershifted with a monoclonal antibody recognizing human TCF-4 (Fig. 3D). Although 293 cells express all four *Tcf/Lef* genes, previous studies showed that TCF-1 and TCF-3 remain associated with insoluble nuclear components and are not eluted from nuclei by conventional extraction procedures (73,74). Thus, we concluded that the second gel-retarded complex contained endogenous LEF-1 protein and we attempted to supershift this complex with an antibody recognizing LEF-1. Addition of the LEF-1-specific antibody to the binding reaction led to almost complete diminishing of the original protein–DNA complex while the TCF-4–DNA complex stayed intact (Fig. 3D). We assumed that the second TCF–DNA complex indeed contains LEF-1 protein, and that the interaction between LEF-1 and the DNA probe was destabilized by the antibody added. Stimulation of 293 cells by Rat2-Wnt-1 cells resulted in the appearance of additional bands that could be further supershifted with a β-catenin antibody (Fig. 3D). Taken together, these data provide clear proof that the exogenous Wnt signal delivered by the Wnt-1-producing feeder cells, is effectively transmitted into human 293 cells, stimulates the formation of TCF/β-catenin complexes and subsequently activates the transcription of Wnt-responsive promoters. We further used this cellular system to study the effects of CtBP1 on Wnt-induced transcription.

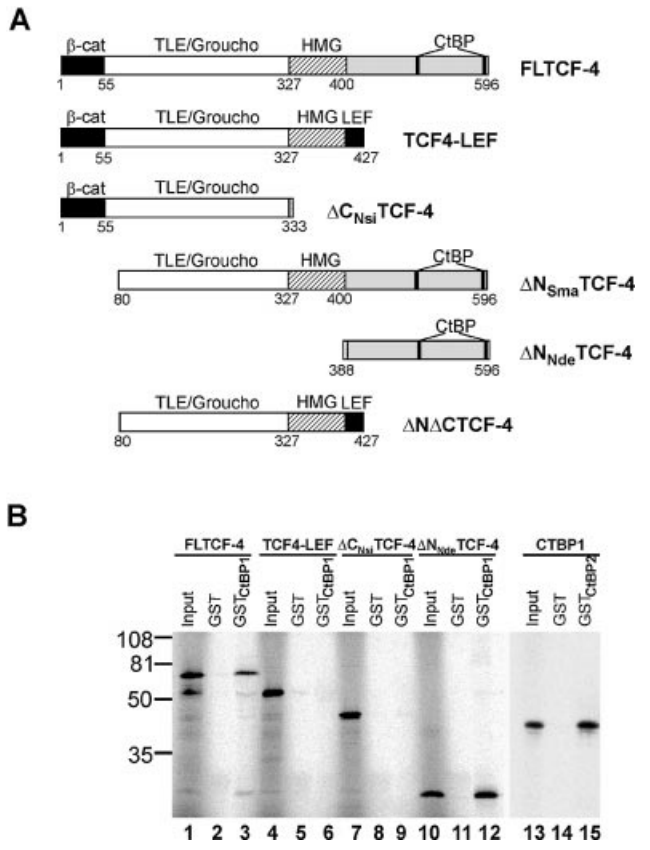

Figure 2. The C-terminus of TCF-4 interacts with CtBP1. (**A**) A schematic representation of the human TCF-4 deletion constructs used in this study. All constructs contained the N-terminal Myc-tag (not depicted). β-cat, β-catenin interaction domain; TLE/Groucho, TLE/Groucho binding domain; CtBP, CtBP-binding sites; HMG, DNA-binding domain; LEF, LEF-1 C-terminus. (**B**) *In vitro* interaction of TCF-4 with CtBP1. GST–CtBP1 and GST–CtBP2 fusion proteins were conjugated to glutathione–Sepharose beads and incubated with the indicated ^{35}S-labeled TCF-4 proteins translated *in vitro*. After washing and recovery of the beads, associated proteins were resolved by SDS–PAGE and analyzed by autoradiography. In lanes 1, 4, 7, 10 and 13, 10% of the input labeled proteins was applied directly onto the gel. Full-length TCF-4 (lane 3) and the TCF-4 C-terminal fragment (lane 12) bind to GST–CtBP1. TCF4–LEF-1 chimera (line 6) or the N-terminal fragment of TCF-4 lacking CtBP-binding sites (lane 9) do not interact with GST–CtBP1. *In vitro* labeled CtBP1 associates with GST–CtBP2 (lane 15). None of the proteins bind to GST-bound Sepharose beads (lanes 2, 5, 8, 11 and 14). The positions of molecular weight markers in kDa are indicated at the left.

CtBP1 represses TCF/β-catenin-dependent transcription by a trichostatin A-sensitive mechanism

CtBP proteins function as short-range transcriptional co-repressors, therefore we examined the functional relevance of the TCF-4–CtBP1 interaction in reporter assays. It has been reported previously that N-terminal modification of the CtBP1 protein may change its association with histone deacetylases (75); for that reason, we tested the activity of three different CtBP1 constructs encoding mCtBP1 with an N-terminal Myc-tag, EGFP-tagged mCtBP1 and, finally, full-length human CtBP1 without N-terminal modification. All three constructs repressed transcription from the Tcf-dependent pTOPFLASH reporter in a concentration-dependent manner in both unstimulated and stimulated 293 cells, whereas the activity of the control reporter pFOPFLASH was not affected by CtBP1 (Fig. 4A, only data for human CtBP1 are shown). CtBP1 was originally described as a protein interacting with the adenoviral oncogene E1A. Since 293 cells express E1A, we used an alternative cell system to avoid the possibility that the observed effects are dependent on E1A expression. We utilized the previously established transient β-catenin–Tcf reporter assay. In this assay, co-transfection of TCF-4 and β-catenin into COS-7 cells results in transactivation of the Tcf-dependent luciferase reporter gene (Fig. 4B). Upon addition of increasing amounts of CtBP1, the luciferase activity decreases proportionally only when the construct encoding full-length TCF-4 (FLTCF-4) capable of binding to CtBP was co-transfected. Transactivation mediated by β-catenin and C-terminally truncated TCF-4 (TCF4–LEF) was not affected by CtBP1. The reporter gene assays indicated that Tcf/β-catenin transcriptional activity is down-regulated by TCF-4–CtBP1 interaction. To study the mechanisms of CtBP1 action in greater detail, we generated 293 cells with inducible expression of EGFP-tagged mCtBP1. We obtained four polyclonal cell lines (293-EGFP–CtBP1/Dox) producing EGFP–mCtBP1 protein in the quantities representing approximately one-half of the amount of endogenous CtBPs (Fig. 5A). For further analysis we utilized a mixture of these cell lines. We further compared EGFP–CtBP1 production from the *CtBP1* transgene with the EGFP–CtBP1 levels obtained in transient transfections. Results of a western blot analysis showed that transfection of 0.5 μg of the CtBP1 expression construct generated cellular amounts of EGFP–CtBP1 protein similar to the levels obtained in 293-EGFP–CtBP1/Dox cells upon removal of doxycycline. Transfection of a larger quantity of the construct produced even more EGFP–CtBP1 protein than the amount of endogenous CtBPs (Fig. 5A). Nevertheless, the relatively moderate expression of the *EGFP–CtBP1* transgene significantly decreased pTOPFLASH transcription in Wnt-1-stimulated cells, whereas the activities of this reporter in 293-EGFP–CtBP1/Dox cells growing in the presence of doxycycline, i.e. with repressed CtBP1, were comparable to values obtained in the parental cell line (Fig. 5B). We readily obtained almost identical amounts of TCF/β-catenin complexes in Wnt-1-stimulated 293-EGFP–CtBP1/Dox cells independently of the expression status of the *CtBP1* transgene (not shown). This indicates that CtBP levels do not interfere directly with the formation of such complexes or with the upstream steps of Wnt signaling. We further determined whether the mechanism of CtBP1 repression relies on histone deacetylases. As shown in Figure 5C, treatment with trichostatin A, a histone deacetylase inhibitor, alleviated the repressive effect of CtBP1 in 293-EGFP–CtBP1/Dox cells. The trichostatin A treatment also completely neutralized the repressive effect of CtBP1 in transient transfection assays (Fig. 5D). We conclude that CtBP1 acts as a repressor of Wnt-mediated transcription via recruitment of histone deacetylases to the target promoter.

Besides CtBP, 293 cells express other TCF-4-interacting proteins, the TLE/Groucho co-repressors (52). Therefore, we asked next whether both types of negative regulators utilize the same inhibitory mechanisms. We examined the impact of different TCF-4 deletions on the activity of the pTOPFLASH reporter in unstimulated and stimulated 293 cells (see Fig. 2A

for details about the constructs used). TCF-4 constructs, when overexpressed in 293 cells, decreased the basal and the Wnt-1-primed activity of pTOPFLASH but, strikingly, TCF4-LEF, which interacts only with TLE/Groucho and lacks the C-terminal CtBP-binding domain, appeared to be more potent repressor than full-length TCF-4 (Fig. 5E). In addition, only repression mediated by full-length TCF-4, i.e. by a protein containing both TLE/Groucho and CtBP interaction regions, has been almost completely alleviated by the trichostatin A treatment. These results indicate that the TLE/Groucho proteins may utilize different mechanisms than do CtBPs to repress the Tcf-responsive promoter and/or that the action of these repressors might be mutually exclusive.

CtBP1 down-regulates the expression of Axin2, a Tcf/β-catenin target in 293 cells

We examined the expression levels of several human genes related to Wnt signaling in Wnt-1-stimulated 293 cells. We designed sets of human-specific primers that selectively amplified cDNA produced from 293 mRNA and did not function with rat or mouse cDNAs, respectively (see Materials and Methods). This allowed us to assess the level of expression of the selected human genes even in 293/Rat2 mixed RNA samples. Upon Wnt-1 stimulation, we noticed significant changes in expression of several genes: (i) we detected a moderate up-regulation of presumptive Wnt targets *Cyclin D1*, *Tcf-1* and *Lef-1* (see Supplementary Material) and (ii) we also detected strong transcriptional activation of *Axin2/Conductin*. Axin2 was recently identified by DNA-array technology as a gene positively regulated by TCF/β-catenin complexes in colorectal and liver tumors and in rat RK3E epithelial cells (76,77). We tested the time course of induction of the *Axin2* transcript in stimulated cells (Fig. 6A). We detected an 8-fold increase in *Axin2* mRNA as early as 3 h after stimulation (a 3 h interval was a minimal time period necessary for attachment of feeder cells onto the 293 cells). The maximal quantity of *Axin2* mRNA was reached after 9 h of co-cultivation. Following this time point, *Axin2* levels started to decrease but, nevertheless, after 15 h still remained approximately six times higher than in control 293 cells. Expression of the *Axin1* gene, an *Axin2* homolog, did not change significantly at any time point tested. We further

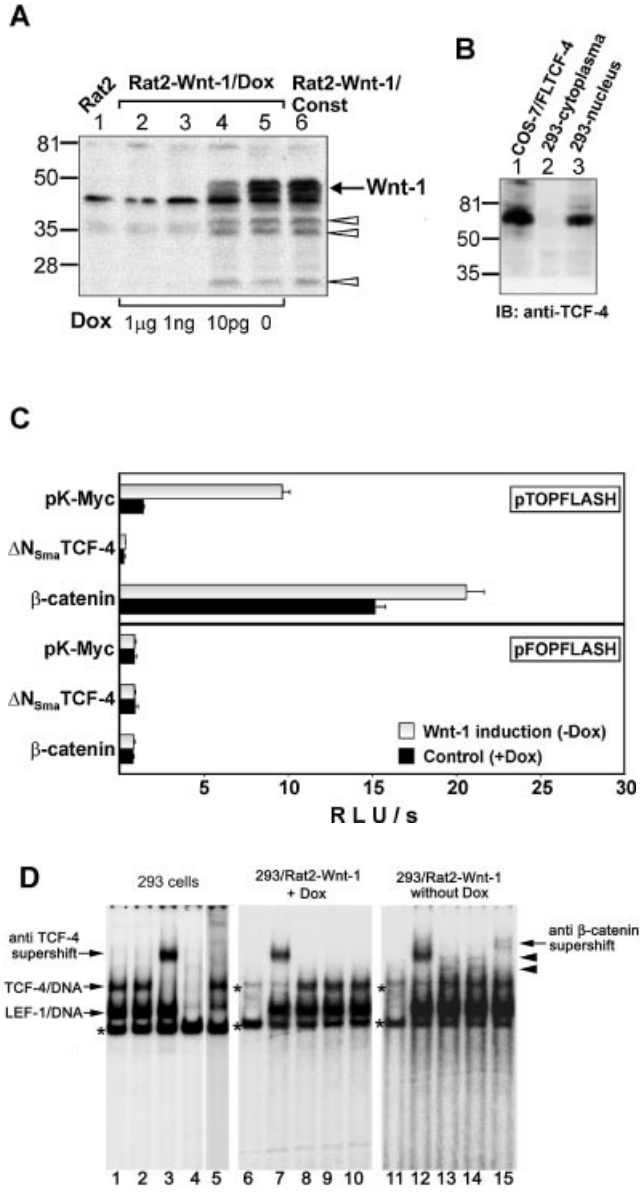

Figure 3. Wnt-1 protein-producing Rat2 feeder cells activate Wnt signaling in 293 cells. (**A**) Wnt-1 expression is strictly regulated in Rat2 fibroblasts by doxycycline. Western blot analysis of total cell lysates from parental Rat2 cells (lane 1), Rat2 cells with doxycycline-regulated Wnt-1 expression (Rat2-Wnt-1/Dox, lanes 2–5) and Rat2 cells with constitutive production of Wnt-1 (Rat2-Wnt-1/Const, lane 6). The Rat2-Wnt-1/Dox cells were grown at different concentrations (1 μg, 1 ng and 10 pg per ml) or in the absence of doxycycline, as indicated at the bottom. SDS–PAGE and immunoblotting with Wnt-1 antibody resolved cell lysates. The arrow shows the position of the putative Wnt-1 protein; the positions of shorter degradation products are indicated by open arrowheads. Molecular weight markers in kDa are at the left. (**B**) 293 cells express a longer form of TCF-4 protein. A total cell lysate from COS-7 cells transfected with expression vector encoding full-length TCF-4E protein (lane 1), and from cytoplasmic and nuclear fractions prepared from 293 cells (lanes 2 and 3, respectively) were resolved by SDS–PAGE and immunoblotted with an anti-TCF-4 antibody recognizing an epitope proximal to the HMG box. Molecular weight markers in kDa are at the left. (**C**) Wnt-1 expressing Rat2 cells induce the activation of the Tcf reporter in 293 cells. The human embryonic kidney 293 cell line was co-transfected with the expression plasmids indicated on the *y*-axis and the Tcf reporter construct pTOPFLASH (containing wild-type Tcf-binding sites) or pFOPFLASH (with mutated Tcf motifs) as a negative control. Four hours post-transfection, DNA mixtures were removed and Rat2-Wnt-1/Dox fibroblasts were subsequently plated over the 293 cells. Cultures were further grown either in the absence (Wnt-1 induction) or presence of doxycycline (control) for 15 h, then the cells were harvested together and processed to assay the reporter gene activities. Luciferase activities [shown on the *x*-axis as relative light units per second (RLU/s)] were corrected for the efficiency of transfection using the internal control *Renilla* pRL-SV40 expression plasmid. Average values and their standard deviations from three independent experiments are shown. (**D**) Wnt-1 induces nuclear TCF–β-catenin complexes in 293 cells. A gel retardation assay performed with nuclear extracts from parental 293 cells (left) and from 293 cells co-cultivated with Rat2-Wnt-1/Dox cells growing in the presence (control, middle) or absence (Wnt-1 induction, right) of doxycycline. Samples in lanes 1, 8 and 13 were incubated under standard conditions. Anti-β-catenin antibody was added to the samples in lanes 2, 10 and 15. Anti-TCF-4 antibody was added to the samples in lanes 3, 7 and 12. Anti-LEF-1 antibody was added to the sample in lane 5. Filled arrowheads indicate the positions of the TCF/β-catenin complexes. A control antibody (anti-HA tag) was added to the samples in lanes 9 and 14. Asterisks indicate non-specific bands also observed with a probe mutated in the Tcf-binding site (lanes 4, 6 and 11).

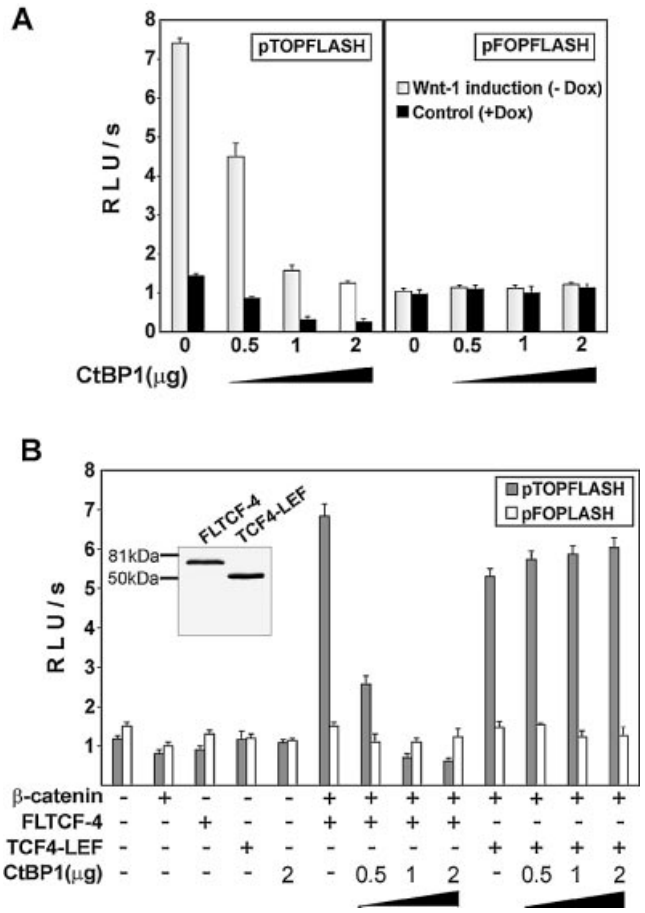

Figure 4. CtBP1 represses TCF/β-catenin transcription. (**A**) CtBP1 represses TCF/β-catenin transcription in 293 cells. Human 293 embryonic kidney cells were co-transfected with the indicated amounts of CtBP1 expression plasmid and the Tcf reporter construct pTOPFLASH or the negative control reporter pFOPFLASH using the lipofectamine reagent. Four hours post-transfection, DNA–lipofectamine mixtures were removed and 293 cells were covered with Rat2-Wnt-1/Dox fibroblasts containing the *Wnt-1* gene driven by the doxycycline-repressed promoter. The cultures were further grown in the presence (control) or absence of doxycycline (Wnt-1 stimulated). Following co-cultivation for 15 h, the cells were harvested, and luciferase (firefly) and *Renilla* luciferase activities were determined in cell lysates. (**B**) CtBP1 can repress transactivation mediated by TCF-4 and β-catenin in COS-7 cells. COS-7 cell line was co-transfected with the Tcf reporter constructs and a specific TCF-4 construct, β-catenin and with the indicated amount of CtBP1 plasmid. Luciferase and *Renilla* luciferase activities were determined in cell lysates 15 h following transfection. Whole cell extracts were analyzed by western blotting with TCF-4 monoclonal antibody (inset). All transfections were done in triplicate. Relative luciferase light units per second (RLU/s) are average values corrected for the efficiency of transfection by determining the luciferase/*Renilla* ratio.

examined the influence of CtBP1 overexpression on the activation of the *Axin2* gene. *Axin2* mRNA in 293-EGFP–CtBP1/Dox cells stimulated by Rat2-Wnt-1/Const feeder cells and with the repressed *EGFP–CTBP1* transgene was up-regulated 6-fold (compared to the same cells co-cultivated with parental Rat2 fibroblasts). However, the induction of *CtBP1* in Wnt-1-activated cells significantly down-regulated *Axin2* expression (Fig. 6B). These results indicate that a cellular dose of CtBPs modulates the activity of the Axin-2 promoter and, by implication, might selectively regulate the expression of other Wnt target genes.

DISCUSSION

In this report we provide evidence for an interaction between the TCF-4 protein, a member of the Tcf/Lef family of nuclear mediators of Wnt signaling, and the transcriptional co-repressor CtBP1. Using Wnt-1-stimulated human embryonic kidney 293 cells, we demonstrate that CtBP1 represses the transcriptional activity of a Tcf/β-catenin-dependent synthetic promoter and, furthermore, decreases the expression of the endogenous Wnt target Axin2. We further show that the inhibitory effect of CtBP is neutralized by the histone deacetylase inhibitor trichostatin A.

TCF/LEF proteins represent a specific type of transcription factor. They harbor β-catenin and TLE/Groucho interaction domains that determine whether, under given circumstances, the protein will act as a transcriptional activator or repressor. TCFs display essentially identical DNA-binding specificities, thus they have been considered as equivalent nuclear components of the Wnt signaling pathway. However, recent studies revealed functional differences among TCF family members. The most obvious feature of individual TCFs is their ability to associate with various cellular proteins. From that point of view, LEF-1 and TCF-3 proteins have been the most thoroughly studied Tcf/Lef family members (see Introduction). The main focus of this study is TCF-4 protein.

The limited solubility of Wnt proteins is a critical obstacle to studying the biological function of these molecules *in vitro*. We developed a dual cell system for Wnt pathway activation in which the target cells are stimulated by cells producing Wnt factors and growing in close proximity. This type of stimulation presumably corresponds well to the situation occurring *in vivo* in solid tissues and organs. At first, we generated rat fibroblast cell lines with constitutive or inducible Wnt-1 expression, and, using a Tcf/β-catenin-dependent reporter assay and a β-catenin supershift assay, we showed that these cell lines evidently activate Wnt signaling in neighboring 293 cells. β-Catenin overexpression from a transiently transfected construct resulted in a robust activation of the Tcf/β-catenin-dependent reporter, which was further augmented if the cells were simultaneously stimulated by Wnt-1-producing feeders (Fig. 3C). This indicates that β-catenin is indeed a key and also a limiting molecule in Wnt signaling. We further utilized this cell system to test the function of CtBP1 in the context of a Wnt-responsive promoter. Since 293 cells express E1A protein and CtBP1 was originally described as a protein interacting with this adenoviral oncoprotein, we used the COS-7 cell system in parallel to avoid the possibility that the observed effects are dependent on E1A expression. The repressive effect of CtBP1 depended directly on the expression levels obtained from either transiently or steadily transfected CtBP1 plasmids. This dosage-dependent repression corresponds well to the phenotype of mice harboring various combinations of *CtBP1* and *CtBP2* mutant alleles. *CtBP1*-null mice are small but viable, whereas *CtBP2* mutants die by E10.5 due to aberrant extraembryonic development. Different *CtBP1/CtBP2* compound mutant mice display additional dose-sensitive defects in a wide range of developmental processes (78). It is surprising that although 293 cells produce an abundance of LEF-1 (79), which is a protein that does not interact with CtBP, higher amounts of CtBP1 efficiently suppressed transcription from the Wnt-responsive

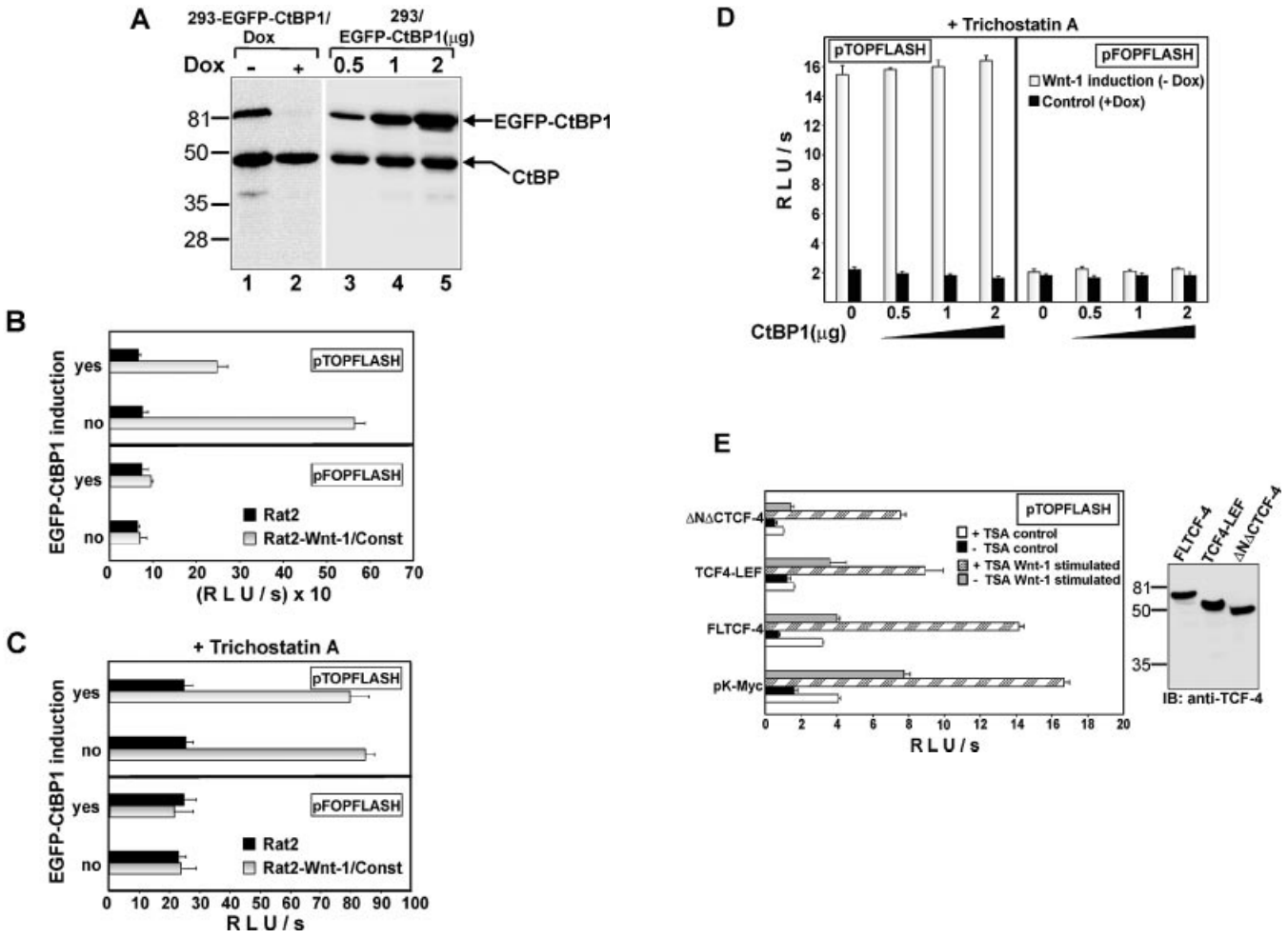

Figure 5. Histone deacetylases inhibitor trichostatin A alleviates the repressive effect of CtBP1 on the Wnt-responsive promoter. (**A**) 293-EGFP–CtBP1/Dox cells produce EGFP-tagged mCtBP1 in quantities that represent one-half of the amount of endogenous CtBPs. Total cell lysates from 293 cells expressing EGFP–CtBP1 from a doxycycline-repressive promoter growing in the absence (EGFP–CtBP1 induced, lane 1) or presence (EGFP–CtBP1 repressed, lane 2) of doxycycline (1 μg/ml) and from the parental 293 cell line transiently transfected with different amounts of plasmid encoding EGFP-tagged CtBP1 (lanes 3–5, micrograms of transfected construct are indicated on the top) were analyzed by western blot analysis. The anti-CtBP monoclonal antibody used to visualize proteins on the blots recognizes both human CtBP1 and CtBP2 proteins. The positions of molecular weight markers in kDa are indicated at the left. (**B**) Expression of the *EGFP–CtBP1* transgene down-regulates activity of the Wnt-resposive promoter in 293-EGFP–CtBP1/Dox cells. 293-EGFP–CtBP1/Dox cell line was transfected with the indicated Tcf reporter constructs. Four hours post-transfection, DNA mixtures were removed, the cells washed extensively and Rat2-Wnt-1/Const fibroblasts (steadily producing the Wnt-1 protein) or parental Rat2 cells were subsequently plated over the 293 transfectants. Cultures were further grown for 15 h either in the absence (EGFP–CtBP induction: yes) or presence (EGFP–CtBP induction: no) of doxycycline (1 μg/ml). (**C**) Trichostatin A treatment releases the repressive function of EGFP–CtBP1. The experiment was performed as described above in (B) except that trichostatin A (final concentration 300 nM) was added simultaneously with the feeder cells. (**D**) Trichostatin A treatment alleviates the CtBP1-mediated repression in transient transfection assay. Human 293 embryonic kidney cells were co-transfected with the indicated amounts of CtBP1 expression plasmid and the Tcf reporter constructs using the lipofectamine reagent. Four hours post-transfection, DNA–lipofectamine mixtures were removed and 293 cells were covered with Rat2-Wnt-1/Dox fibroblasts. The cultures were further grown in the presence (control) or absence of doxycycline (Wnt-1 stimulated). Trichostatin A (300 mM final) was added simultaneously with the feeder cells. Following co-cultivation for 15 h, the cells were harvested and luciferase and *Renilla* luciferase activities were determined in cell lysates. (**E**) Tcf-mediated repression is partially insensitive to trichostatin A. Human 293 embryonic kidney cells were co-transfected with empty expression vector pK-Myc or TCF-4 deletion constructs indicated on the *y*-axis and the Tcf reporter plasmid pTOPFLASH. TCF-4 proteins were produced at comparable levels in the transfected cells as shown by western blot analysis (right panel). Four hours post-transfection, cells were covered with Rat2 cells (control) or Rat2-Wnt-1/Const cells (Wnt-1 stimulated), and half of the samples were further treated with trichostatin A (TSA, final concentration 300 nM). Following an additional 12 h, the cells were harvested, and luciferase and *Renilla* luciferase activities were determined in cell lysates. Transfections were done in triplicate. Average luciferase light units per second (RLU/s) corrected to *Renilla* luciferase activities and their standard deviations are shown.

reporter to basal levels (Fig. 4A). This fact might be explained by the greater ability of TCF-4 to form a ternary complex with DNA and β-catenin (80).

TCF-4 (and TCF-3) resembles two other repressors, Hairy and Brinker, which also interact with both TLE/Groucho and CtBP proteins (81,82). The precise mechanisms of how TLE/Groucho and CtBP contribute to gene repression remain to be elucidated. In *Drosophila*, Groucho functionally interacts with the histone deacetylase Rapd3 (51). Recruitment of Rapd3 to target promoters would result in the formation of a more compact chromatin structure followed by a transcriptionally repressed state. The recruitment of histone deacetylases is likely to be the main function of the TLE/Groucho proteins, although only the partial release of TLE/Groucho-Rapd3

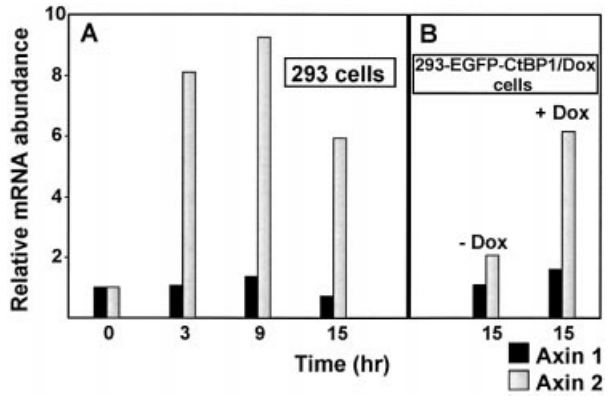

Figure 6. CtBP1 down-regulates the expression of Axin2 in 293 cells. (**A**) Wnt-1 activates *Axin2* mRNA in 293 cells. The results of quantitative real-time PCR performed with cDNA generated from 293 cells stimulated by Wnt-1-producing feeder cells (Rat2-Wnt-1/Const) or by control Rat2 cells are shown. The 293 and feeder cells were co-cultivated for the indicated period of time, then harvested and random primed cDNA was prepared from total RNA. The PCR reactions were performed for each primer set in triplicate using cDNAs produced from at least two independent RNA isolates. (**B**) EGFP–CtBP1 decreases responsiveness of the *Axin2* gene to the Wnt-1 stimulation but has no effect on the expression of the *Axin1* gene. 293-EGFP–CtBP1/Dox cells were co-cultivated with feeder cells stably producing Wnt-1 protein (Rat2-Wnt-1/Const) or control Rat2 fibroblasts as a negative control. Cells were co-cultivated for 15 h in the presence (EGFP–CtBP1 repressed) or absence (EGFP–CtBP1 overexpressed) of doxycycline (1 μg/ml). The random primed cDNAs generated from the relevant RNAs were analyzed. The PCR reactions were performed for each primer set in triplicate using cDNAs produced from at least two independent RNA isolates. The results of a representative experiment are shown. The results were analyzed using the LightCycler 5.1 software package, and the values of a representative experiment are shown. The relative abundance of *Axin1* and *Axin2* mRNA in Wnt-1-stimulated versus control cells was derived from the average CT values of each triplicate after normalizing to the levels of *SDHA* cDNA.

repression by the histone deacetylase inhibitor trichostatin A indicates that additional mechanisms of repression may exist. CtBP also induces transcriptional silencing in a histone deacetylase-dependent or -independent manner. CtBP1 associates with class I and class II histone deacetylases, and CtBP-related down-regulation of certain promoters has been reported to be sensitive to trichostatin A (83). In contrast, repression of several other promoters was insensitive to trichostatin A treatment (75). We detected a complete release of the CtBP-mediated repression by trichostatin A. This clearly indicates the involvement of the histone deacetylases in the repression mechanism. Since 293 cells express both TLE/Groucho and CtBP proteins (52 and this manuscript), we further evaluated the contribution of the TLE/Groucho and CtBP co-repressors to the repression of the activity of the pTOPFLASH reporter. Overexpression of TCF-4–LEF deletion containing only the TLE/Groucho interaction domain significantly inhibited the basal and Wnt-1-dependent activity of the Tcf reporter. This is in agreement with the observation that the C-terminally truncated forms of XTCF-3 keep their repressive potential in *Xenopus* embryos, even if they lack the CtBP-interacting domains (61). Surprisingly, the putative repressive action of TLE/Groucho was only partly suppressed by trichostatin A, and a greater sensitivity to this chemical was only revealed by the full-length TCF-4-mediated repression (Fig. 5E). This implies the possibility of parallel repressive action of CtBP and TLE/Groucho, which might contribute to the regulation of different Wnt targets. Since the TLE/Groucho and CtBP-binding sites are quite far apart in TCF-4 and TCF-3 proteins, we assume that there is no steric competition for these sites as in Hairy protein (82).

We took advantage of the different origin of human 293 and rat feeder cells and used real-time quantitative RT–PCR to analyze the changes in expression of selected genes upon Wnt stimulation. The strongest up-regulation was observed for *Axin2* mRNA. This finding was rather expected because Axin2 was recently identified as a gene activated by β-catenin overexpression in the related rat RKE3 cells (76). We further evaluated the impact of CtBP1 overexpression on Axin2 activation and detected a strong reduction in the levels of *Axin2* mRNA. Such a robust effect was surprising because the quantities of the ectopically expressed CtBP1 protein represented approximately one-half of the amount of endogenous CtBPs. 293 cells express all four TCF/LEF proteins, although at different levels, TLE/Groucho, and both CtBP1 and CtBP2 co-repressors (V. Korinek, unpublished data). TCF-4 associates with TLE/Groucho proteins and also with CtBPs, and these proteins also form homo- or heterodimers. The strength of the interaction between CtBPs and their partners can be further potentiated by the levels of nuclear NAD(H) (84,85). We propose that the composition of various Wnt signaling pathway components and their expression levels dictate the primary sensitivity of a given cell to the Wnt signal. The balance in such a system can be disturbed by changing the levels of only one component. Likewise the defects in other negative regulators of Wnt signaling (e.g. in the tumor suppressor APC), mutations that inactivate CtBP proteins could also lead to inappropriate activation of the Wnt pathway. On the other hand, non-physiological increases in the expression levels of CtBPs might by implication result in a significant reduction in Wnt signaling output in the stimulated cell.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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SUPPLEMENTARY MATERIAL

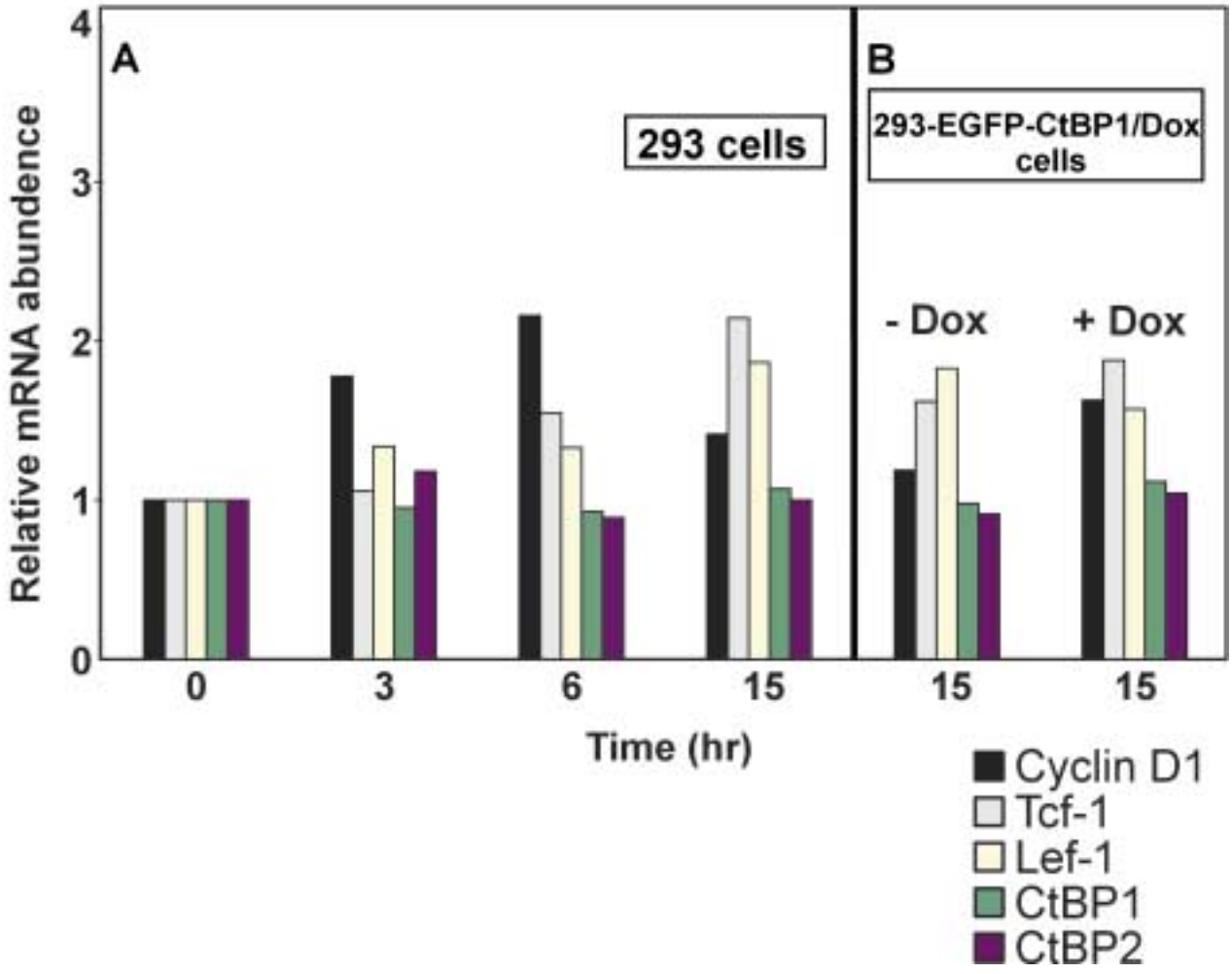
Wnt-1 upregulates Wnt-targets *Cyclin D1*, *Lef-1* and *Tcf-1* mRNA in 293 cells

A, Results of quantitative real-time PCR performed with cDNA generated from 293 cells stimulated by feeder cells stably producing Wnt-1 protein (Rat2-Wnt-1/Const) or by control Rat2 cells are shown. The 293 and feeder cells were co-cultivated for the indicated period of time, then harvested, and random primed cDNA was prepared from total RNA.

B, CtBP1 overexpression has no obvious effect on the levels of *Cyclin-1*, *Lef-1*, *Tcf-1*, *CtBP1,* and *CtBP2* RNA in Wnt-1-stimulated 293 cells. 293-EGFP-CtBP1/Dox cells were co-cultivated with Rat2-Wnt-1/Const or control Rat2 fibroblasts as a negative control for 15 hours in the presence (EGFP-CtBP1 repressed) or absence (EGFP-CtBP1 over-expressed) of Doxycycline (1 μg/ml). The random primed cDNAs generated from the relevant RNAs were analysed. The picture shows the relative abundance of *Cyclin D1, Lef-1, Tcf-1, CtBP1* and *CtBP2* mRNAs in Wnt-1-stimulated 293-EGFP-CtBP1/Dox cells versus control 293-EGFP-CtBP1/Dox in two situations: (1) when EGFP-CtBP1 is expressed (-Dox) or (2) repressed (+Dox).

The results were analyzed using the LightCycler 5.1 software package, and the values of a representative experiment are shown. The relative abundance of *Cyclin-1*, *Lef-1*, *Tcf-1*, *CtBP1* and *CtBP2* mRNA in Wnt-1-stimulated versus control cells was derived from the average CT values of each triplicate after normalizing to the levels of *SDHA* cDNA.

Figure 6 -supplementary data



3.2 HIC1 snižuje aktivitu signalizace Wnt prostřednictvím strhávání TCF-4 a β-kateninu do jaderných tělísek

Několik prací publikovaných v posledních letech ukázalo, že represivní aktivita zprostředkovaná proteiny CtBP1 a CtBP2 může být výrazně ovlivněna změnami v jejich vnitrobuněčné lokalizaci. Proteiny CtBP se mohou nacházet jak v cytoplazmě, tak v buněčném jádře, kde se podílejí na represi transkripce. Vliv na lokalizaci těchto proteinů mohou mít proteinové modifikace. Zatímco fosforylace prostřednictvím Pak1 kinázy relokuje CtBP1 do cytoplazmy, sumoylace je určující pro jadernou lokalizaci CtBP1 [205-207]. Distribuce CtBP může být rovněž ovlivněna přímou interakcí s proteiny schopnými vázat CtBP. Protein nNOS zadržuje CtBP1 v cytoplazmě, naproti tomu protein HIC1 relokuje CtBP1 do specifických jaderných tělísek (Hic1 bodies) [208, 209]. Přepokládali jsme, že změny v lokalizaci proteinu CtBP1 by mohly ovlivňovat jeho represivní aktivitu na transkripci mediovanou TCF-4/β-kateninem. Proto jsme se v následující práci zaměřili na působení proteinu HIC1.

HIC1 (Hypermethylated In Cancer 1) byl identifikován jako potenciální nádorový supresor, jehož gen je často epigeneticky inaktivován prostřednictvím methylace promotorové oblasti, a to v řadě různých typů nádorů. [210]. Gen *HIC1* kóduje transkripční faktor ze skupiny proteinů BTB/POZ (Broad-complex, Tramtrack, Bric à brac/Poxvirus, and Zinc finger). Protein HIC obsahuje na svém N-konci doménu BTB/POZ, která je zodpovědná za multimerizaci a interakce s ostaními proteiny. Multimerizce proteinu HIC1 se projevuje morfologicky v podobě specifických distinktních jaderných struktur. V centrální části proteinu HIC1 je lokalizován specifický motiv GLDLSKK, jenž zprostředkovává vazbu s proteiny CtBP. C-konec obsahuje několik DNA-vazebných motivů typu "Zn-finger" . [209, 211, 212]. Experimenty s aberantní inaktivací genu *HIC1* u myší potvrdily jeho roli jako buněčného tumorového supresoru. Zatímco myši $Hic1^{-/-}$ umírají v průběhu embryonálního vývoje nebo perinatálně, tak u heterozygotů $Hic1^{+/-}$ se v dospělosti objevuje řada maligních spontánních tumorů, které jsou pohlavně specifické. Pro tyto tumory je charakteristická silná methylace zbývající alely *Hic1*, která vede k úplné absenci exprese *HIC1* v těchto nádorech. [213]. Gen *HIC1* je lokalizován v oblasti chromozomu 17p13.3, která je často deletována u pacientů s Miller-Diekerovým syndromem. K projevům tohoto syndromu patří řada mozkových a obličejových defektů, které jsou prakticky totožné s defekty u myší $Hic1^{-/-}$ [214]. Aktivita proteinu HIC1 je spojena s aktivitou jiného tumor supresoru – p53. HIC1 tvoří transkripčně represivní

komplex s histon deacetylázou SIRT1, který reprimuje transkripci genu *SIRT1*. V normálních buňkách je tak hladina proteinu SIRT1 udržována na nízké úrovni. V řadě nádorů, u nichž je gen *HIC1* epigeneticky inaktivován, je naopak úroveň exprese *SIRT1* zvýšená. Deacetyláza SIRT1 je schopna deacetylovat tumor supresor p53 a způsobuje tak jeho inaktivaci. V normálních buňkách je navíc protein p53 schopen stimulovat expresi *HIC1*. Vztah mezi p53 a HIC1 je tedy typickým příkladem autoregulační smyčky. [215-218].

V naší práci jsme potvrdili, že HIC1 je schopen relokovat CtBP1 do specifických nukleárních struktur. V případě koexprese *HIC1* a *CtBP1* současně s *TCF-4*, dochází ke změně lokalizace TCF-4 a transkripční faktor TCF-4 je rovněž relokován do jaderných tělísek. HIC1 je schopen změnit lokalizaci TCF-4 v buněčném jádře nezávisle na CtBP1, jak jsme ukázali v buňkách CtBP(-/-), které mají prostřednictvím rekombinace inaktivovány geny *CtBP1* a *CtBP2*. K podobnému závěru vedly experimenty založené na expresi různých mutantních forem *TCF-4* a *HIC1*. Protein CtBP1 má ovšem v procesu relokace TCF-4 důležitou strukturní roli a je významný pro efektivitu této relokace. V buňkách, v nichž je *CtBP1* expremován, způsobuje HIC1 takřka úplnou relokaci TCF-4, na rozdíl od buněk CtBP(-/-), kde je relokována jen určitá část TCF-4.

Protein HIC1 přímo interaguje s TCF-4 a to jak *in vitro* tak *in vivo*. Koimunoprecipitace provedená z extraktů buněk CtBP(-/-) navíc potvrdila, že zprostředkující role CtBP pro interakci mezi HIC1 a TCF-4 není v buňkách nutná.

HIC1 výrazným způsobem redukuje úroveň transkripce mediované komplexy Tcf/β-katenin. Represivní aktivita HIC1 byla prokázána pomocí dvou různých luciferázových reportérů obsahujících buď syntetický Lef/Tcf responsibilní promotor, nebo promotor genu *Axin2*, který je cílovým genem signalizace Wnt. V několika buněčných liniích jsme prokázali schopnost nádorového supresoru HIC1 blokovat transkripci cílových genů signalizace Wnt. Potvrzením represivní funkce HIC1 byly rovněž výsledky experimentů založených na nefyziologickém snížení exprese HIC1 pomocí RNA-interference v primárních (nenádorových) buněčných liniích. Tyto buňky přirozeně exprimují HIC1 a ten je, stejně jako v případě nefyziologicky exprimovaného proteinu, lokalizován ve specifických nukleárních strukturách. Snížení exprese HIC1 vede nejen k vymizení těchto struktur z buněčného jádra, ale zvyšuje transkripční aktivitu Tcf-specifického genu *Axin2* u buněk stimulovaných ligandem Wnt3a. Při studiu exprese cílových genů signalizace Wnt jsme potvrdili, že jejich exprese je silně závislá na buněčném typu. Pokud byly buňky stimulovány ligandem Wnt3a, pak došlo u buněk HEK293 k aktivaci transkripce genů *Sp5*,

Axin2 a *CyklinD1*. Stejný typ aktivace vedl naproti tomu u primárních buněk pouze k transkripci genu *Axin2*.

HIC1 by mohl inhibovat transkripci tak, že by svojí interakcí s TCF-4 bránil vazbě β-kateninu na transkripční faktor TCF-4, a tím by blokoval tvorbu transkripčně aktivních heterokomplexů TCF-4/β-katenin. Jak jsme ukázali v případě buněk DLD-1, u kterých je díky mutaci proteinu APC signalizace Wnt konstitutivně aktivní, HIC1 tímto způsobem transkripci nereprimuje. Pokud je HIC1 v těchto buňkách exprimován, je lokalizován v komplexu společně s TCF-4 a β-kateninem. Přesto v těchto buňkách HIC1 účinně inhibuje transkripci Wnt-specifického genu *Tenascin C*. Jiným možným způsobem působení represoru HIC1 by mohla být jeho přímá represivní aktivita na promotorech cílových genů Wnt-signalizace. HIC1 je schopen přímo inhibovat transkripci, jak bylo ukázáno v případě genu *SIRT1*. Je rovněž známo, že jiný člen rodiny transkripčních faktorů BTB/POZ protein Kaiso interaguje přímo se sekvenčně specifickými elementy cílových genů signalizace Wnt. Chromatinová imunoprecipitace ovšem ukázala, že protein HIC1 není přímo ani nepřímo (tzn. prostřednictvím TCF-4) asociován s promotorovými oblastmi Wnt-specifických genů. Na druhou stranu pokud je v buňkách DLD-1 exprimován HIC1 dochází k výrazné redukci v množství β-kateninu a TCF-4, které jsou asociovány s promotorem Wnt-specifického genu *TenascinC*. Podobné výsledky jsme získali v případě genu *Sp5* u buněk HEK293 v nichž byl ektopicky exprimován HIC1 a β-katenin.

Mechanismus represivního působení tumor supresoru HIC1 na transkripci Wnt-specifických genů je tedy pravděpodobně založený na relokaci komplexů Tcf/β-katenin do již zmíněných specifických nukleárních struktur, které jsou prostorově a funkčně vzdálené od míst, kde dochází k transkripci Wnt-specifických genů. Tato relokace je umožněna přímou interakcí mezi proteiny HIC1 a TCF-4. β-katenin je mimo specifické promotorové oblasti relokován pravděpodobně prostřednictvím TCF-4, ačkoliv se nedá vyloučit existence zatím neznámého faktoru, který by tento proces medioval nezávisle na TCF-4. Strukturní funkce proteinu CtBP může zvýšit efektivitu této relokace, a tím také efektivitu transkripční represe způsobenou proteinem HIC1.

Vzájemná interakce mezi efektory signalizace Wnt a proteinem HIC1 může být významná v procesu nádorové transformace. Epigenetická inaktivace genu *HIC1* v buňkách s nefyziologicky aktivovanou Wnt-signalizací může přispět spolu se ztrátou aktivity tumor supresoru p53 ke vzniku nádorů.

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HIC1 attenuates Wnt signaling by recruitment of TCF-4 and β-catenin to the nuclear bodies

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The hypermethylated in cancer 1 (*HIC1*) gene is epigenetically inactivated in cancer, and in addition, the haploinsufficiency of *HIC1* is linked to the development of human Miller–Dieker syndrome. *HIC1* encodes a zinc-finger transcription factor that acts as a transcriptional repressor. Additionally, the HIC1 protein oligomerizes via the N-terminal BTB/POZ domain and forms discrete nuclear structures known as HIC1 bodies. Here, we provide evidence that HIC1 antagonizes the TCF/β-catenin-mediated transcription in Wnt-stimulated cells. This appears to be due to the ability of HIC1 to associate with TCF-4 and to recruit TCF-4 and β-catenin to the HIC1 bodies. As a result of the recruitment, both proteins are prevented from association with the TCF-binding elements of the Wnt-responsive genes. These data indicate that the intracellular amounts of HIC1 protein can modulate the level of the transcriptional stimulation of the genes regulated by canonical Wnt/β-catenin signaling.

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Introduction

The Wnt signaling pathway plays essential roles in different developmental processes, including cell determination, stem cell survival and organogenesis. In addition, mutational activation of this pathway is implicated in deregulated cell growth and cancerogenesis (reviewed in Logan and Nusse, 2004; Reya and Clevers, 2005). The stabilization of β-catenin is central to the canonical Wnt pathway. In the absence of Wnt signals, β-catenin is phoshorylated by a complex of proteins, including adenomatous polyposis coli (APC), glycogen synthase kinase-3β and axin. Phosphorylation of β-catenin results in its ubiquitylation and degradation by the proteasome. Wnt factors inhibit the APC complex. The result of such inhibition is the stabilization of β-catenin, which accumulates in the cell and translocates into the nucleus where it associates with transcription factors of the TCF/LEF family. TCF/LEF proteins function as nuclear effectors of the Wnt signaling pathway. The DNA-binding specificity of these polypeptides is defined by the HMG box, an 80 amino-acid domain whose primary sequence is virtually identical in all TCF/LEF family members (reviewed in Clevers and van de Wetering 1997). TCF/LEF factors possess only a limited ability to activate transcription. They act as 'connectors' linking other polypeptides to a distinct set of promoters. β-Catenin contains a strong transcription activation domain, thus its interaction with TCFs results in transcription of the Wnt-responsive genes. Many of these genes execute Wnt-mediated cell specification during development or regulate cell proliferation (for more detailed information, refer to the Wnt homepage http://www.stanford.edu/~rnusse/wntwindow.html). In contrast, mammalian TCFs also bind to TLE/Groucho corepressors and, in the absence of the Wnt signal, repress the transcription of TCF-specific promoters (Roose *et al*, 1998). Additionally, two TCF/LEF family members, TCF-3 and TCF-4, associate with C-terminal binding proteins (CtBPs) (Brannon *et al*, 1999; Valenta *et al*, 2003).

The CtBP proteins bind to a short sequence motif PLDLS conserved among the E1A proteins of all human and primate adenoviruses. Different variants of this motif are also present in many other CtBP-interacting partners that function mainly as sequence-specific DNA-binding transcription factors.

In this study, we focused on one of the CtBP-associating proteins, hypermethylated in cancer 1 (HIC1), which redirects CtBP to a specific set of nuclear dot-like structures called HIC1 bodies (Deltour *et al*, 2002). The *HIC1* gene was identified as a candidate tumor suppressor gene frequently epigenetically silenced or deleted in different types of solid tumors (Herman and Baylin, 2003). *HIC1* encodes a zinc-finger transcription factor that belongs to a group of proteins known as the BTB/POZ family (Broad-Complex, Tramtrack, Bric à brac/poxvirus, and zinc finger) (reviewed in Albagli *et al*, 1995). A 714 amino-acid human HIC1 polypeptide contains the N-terminal BTB/POZ domain involved in dimerization and in protein–protein interactions. The C-terminal region interacts with a specific DNA sequence; the GLDLSKK motif responsible for the interaction with the CtBP proteins is located in the central part. Gene inactivation experiments in mice recently confirmed that HIC1 is a genuine tumor suppressor. Heterozygous *Hic1*$^{+/-}$ mice develop malignant spontaneous tumors after a year of life (Chen *et al*, 2003, 2004). These tumors show dense methylation of the remaining wild-type *Hic1* allele promoter accompanied by a complete absence of *Hic1* expression in the cancer tissue. *HIC1* gene resides within a 350 kb region on chromosome 17p13.3, deleted in most patients with Miller–Dieker syndrome (MDS) (Dobyns and Truwit, 1995). This links (in addition to tumorigenesis) the haploinsufficiency of *HIC1* to the development of MDS.

In the present study, we show specific binding between HIC1 and a principal Wnt signaling pathway component, TCF-4. We further demonstrate that overexpression of HIC1

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suppresses the TCF-mediated transcription, and *vice versa*, the inactivation of endogenous HIC1 by RNA interference (RNAi) increases the basal expression of the *Axin2* gene and elevates the transcriptional response of this Wnt signaling target to Wnt stimulation. A deletion mutant of HIC1 lacking the oligomerization BTB/POZ domain can neither form the nuclear bodies, nor antagonize Wnt signaling, nor interact with TCF-4 *in vivo*. This clearly indicates that the HIC1 inhibitory function depends on the ability to form nuclear bodies and to recruit TCF-4 into these structures. Interestingly, β-catenin is also relocated by HIC1, but this sequestration seems to be indirect and mediated via its interaction with TCF-4. In addition, we provide evidence that CtBP1 increases the efficiency of recruitment of the TCF-4 into the HIC1 bodies and further strengthens the suppressive effect of the HIC1 protein on Wnt signaling. Finally, using chromatin immunoprecipitation (ChIP), we show that as a consequence of the relocation into the HIC1 speckles, TCF-4 and β-catenin are prevented from binding to the promoters of the TCF-responsive genes.

Results

HIC1 sequesters TCF-4 into nuclear bodies

Exogenous CtBP1 is distributed in the cytoplasm and in the nucleus in a mostly diffused pattern. Only a fraction of nuclear CtBP1 is localized into distinct structures, described previously as CtBP bodies (Sewalt *et al*, 1999). Cotransfection of the full-length HIC1 construct results in complete relocation of CtBP1 into the nuclear HIC1 bodies (Figure 1B). This relocation is dependent on the direct interaction between HIC1 and CtBP1, as the HIC1-ΔCtBP polypeptide lacking the CtBP-interacting motif loses the translocative properties of the wild-type HIC1 protein. The HIC1 mutant with a deletion encompassing the N-terminal oligomerization BTB/POZ domain still preserved the ability to 'pull' CtBP1 into the nucleus. However, the localization of both proteins was diffuse and no formation of nuclear punctuated structures was observed.

Previously, we have shown an interaction between CtBP1 and TCF-4 in yeast and *in vitro* (Valenta *et al*, 2003). However, we and others in following studies were unable to detect the association of these proteins in mammalian cells. We took advantage of the clear nuclear targeting of CtBP1 by HIC1 and visualized TCF-4 and CtBP1 using confocal microscopy.

In CtBP-positive COS-7 cells, TCF-4 was efficiently sequestered into the HIC1 bodies (Figure 2B). Triple staining and the overlay of the images showed that in these bodies TCF-4, HIC1 and CtBP1 co-localized. CtBP interacts with both, TCF-4 and HIC1 proteins, thus we supposed that the TCF-4 recruitment was mediated by CtBP. As expected, mutated TCF-4 (TCF-4mutCtBP), which is unable to bind CtBP, was not sequestered into the HIC1 bodies even in the *CtBP1* background (Figure 2C). Surprisingly, in *CtBP(−/−)* cells (these cells were derived from $CtBP1^{-/-}CtBP2^{-/-}$ embryos (Hildebrand and Soriano, 2002)), wild-type HIC1 and TCF-4 still partially colocalized in the nuclear dots. We examined next, whether variants of HIC1 differ in their ability to concentrate TCF-4 into the HIC1 bodies in *CtBP1(+)* cells. Whereas wild-type proteins perfectly colocalized in nuclear dots, the HIC1-ΔCtBP mutant displayed only limited capability to sequester nuclear TCF-4.

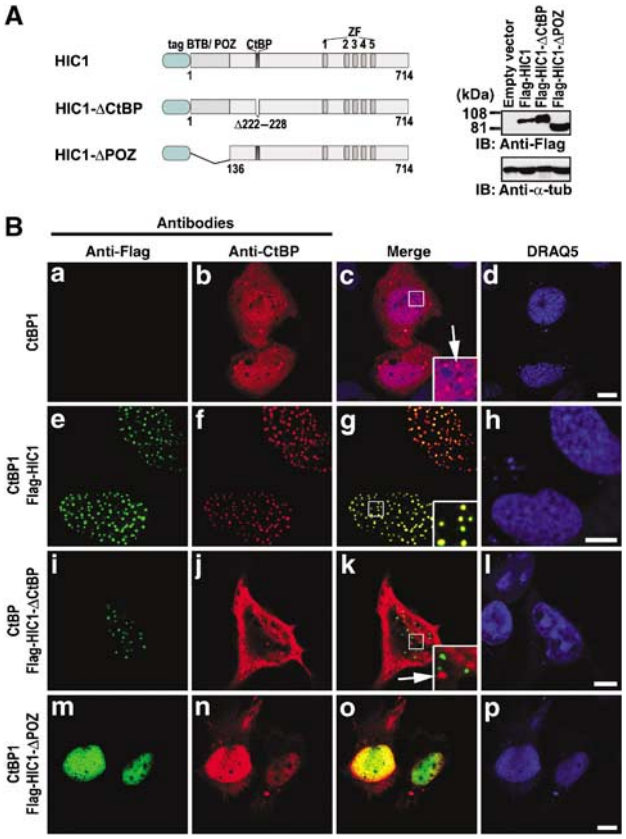

Figure 1 HIC1 targets CtBP into the nuclear bodies. (**A**) A schematic representation of the human HIC1 constructs used in this study. tag, Flag or EGFP tag; BTB/POZ, the BTB/POZ domain; CtBP, CtBP-binding site; ZF, five C_2H_2 Krüppel-like zinc fingers. Right, Western blots of total cell extracts after transfection with the Flag-HIC1 constructs, probed with anti-Flag or with anti-α-tubulin as internal control. (**B**) Confocal microscopy images of *CtBP(−/−)* cells trasfected with constructs indicated on the left and subsequently stained with mouse anti-Flag and rabbit anti-CtBP antibody. The DRAQ5 nuclear stain was gained in the blue channel. Mutant HIC1-ΔCtBP polypeptide lacking the CtBP-interacting motif displays the punctuated expression of the wild-type HIC1 protein (compare (e) and (i)) but does not influence the distribution of CtBP (compare (b) and (j)). As seen in (j, k), a fraction of nuclear CtBP is still localized in the CtBP bodies (arrows in insets (c, k)), which evidently differ from the HIC1 bodies. Bar, 10 μm.

Taken together, these results suggested that TCF-4 and HIC1 could form nuclear aggregates even in the absence of CtBP; nevertheless, CtBP mediates more efficient recruitment of TCF-4 into the HIC1 bodies.

TCF-4 binds directly to HIC1

The existence of HIC1/TCF-4 complexes in mammalian cells was evidenced using co-immunoprecipitation of HIC1 with endogenous TCF-4. By using anti-TCF-4 antibody, a robust coisolation of TCF-4 with wild-type HIC1, and with HIC1-ΔCtBP, was obtained from lysates of human 293 cells; the truncated HIC1-ΔPOZ protein did not co-immunoprecipitate with TCF-4 (Figure 3A). TCF-4 was also isolated from the Flag-tagged HIC1 using anti-Flag or anti-HIC1 antibodies. Furthermore, we immunoprecipitated the endogenous HIC1/TCF-4 complexes from whole-cell lysates prepared from mouse embryos on day 12.5 p.c. We also performed coimmunoprecipitation experiments in mouse *CtBP(−/−)*

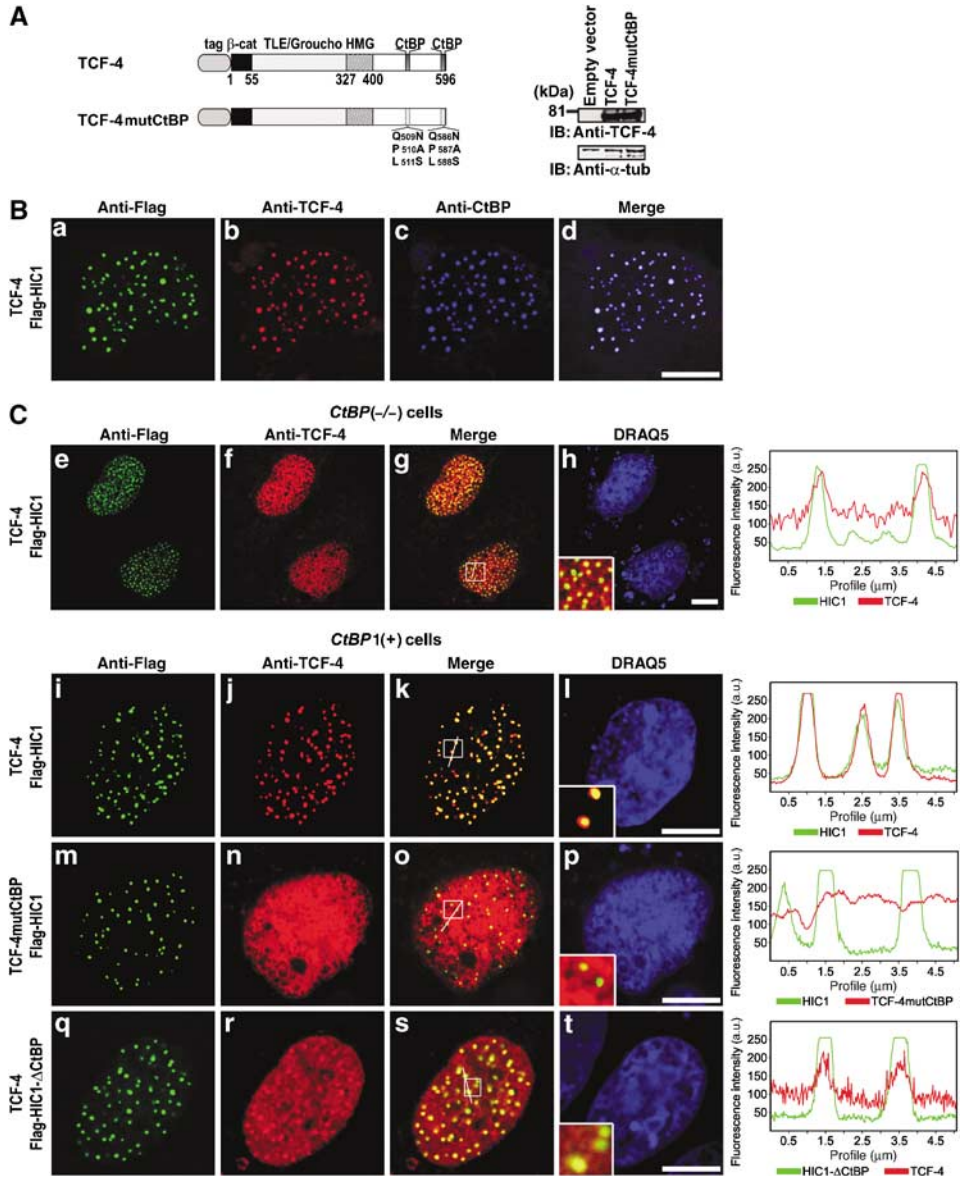

Figure 2 TCF-4 and HIC1 form nuclear protein complexes in mammalian cells. (**A**) TCF-4 expression constructs. The TCF-4mutCtBP polypeptide has a triple amino-acid substitution of each CtBP-binding motif as indicated. tag, myc or EGFP tag; β-cat, β-catenin interaction domain; TLE/Groucho, TLE/Groucho binding domain; CtBP, CtBP binding sites; HMG, DNA-binding domain. Right, Western blots of total cell extracts after transfection with the indicated TCF-4 constructs, probed with anti-TCF-4 or with anti-α-tubulin. (**B**) CtBP, HIC1 and TCF-4 colocalize in COS-7 cells. (**C**) Simultaneous interaction between CtBP, TCF-4 and HIC1 is essential for the efficient nuclear sequestration of TCF-4 into the HIC1 bodies. Confocal microscopy images of *CtBP(−/−)* and *CtBP1(+)* cells transfected with the indicated constructs (left) and stained with anti-Flag and anti-TCF-4 antibody. The right panel shows the overlap of fluorescence intensity peaks along profiles as indicated in the merged micrographs. The nuclear sequestration of TCF-4 by HIC1 is less efficient in *CtBP(−/−)* than in *CtBP(+)* cells (compare (e, f, g, h) to (i, j, k, l)). The formation of the TCF-4/HIC1 bodies in *CtBP(+)* cells strictly depends on the presence of the intact CtBP-binding sites in TCF-4 (m, n, o, p). Notice only a partial colocalization of TCF-4 and HIC1-ΔCtBP (q, r, s, t). Bar, 10 μm.

and *CtBP1(+)* cells, and we detected association of EGFP-tagged HIC1 and TCF-4 in both *CtBP*-negative and *CtBP1*-positive cells. The coimmunoprecipitation was specific for HIC1, because a parallel assay did not show any binding of a control, EGFP-nls protein to TCF-4 (Figure 3B, only results from *CtBP(−/−)* cells are shown). These results implied a direct interaction between TCF-4 and HIC1.

The direct binding between TCF-4 and HIC1 was studied *in vitro* by pull-down assays between bacterially expressed GST-tagged TCF-4 and *in vitro* translated HIC1. GST-TCF-4 associated only with *in vitro* translated full-length HIC1 and not with the N-terminally truncated HIC1-ΔPOZ (Figure 4B). As was shown by others, the deletion of the BTB/POZ domain involved in the homo- and heteromeric interactions often prevents the association of HIC1 with other partners, although this domain is not directly included in the protein–protein interaction (Deltour *et al*, 2002). Due to extensive degradation of GST-HIC1 in bacterial lysates, we could not test the binding of the full-length HIC1 immobilized on the glutathione-Sepharose beads to *in vitro* translated TCF-4. Nevertheless, we prepared GST-fusion proteins containing partly overlapping N-terminal, internal, and C-terminal HIC1 fragments (Figure 4A), and used these more stable proteins in pull-down assays. As shown in Figure 4B, all three

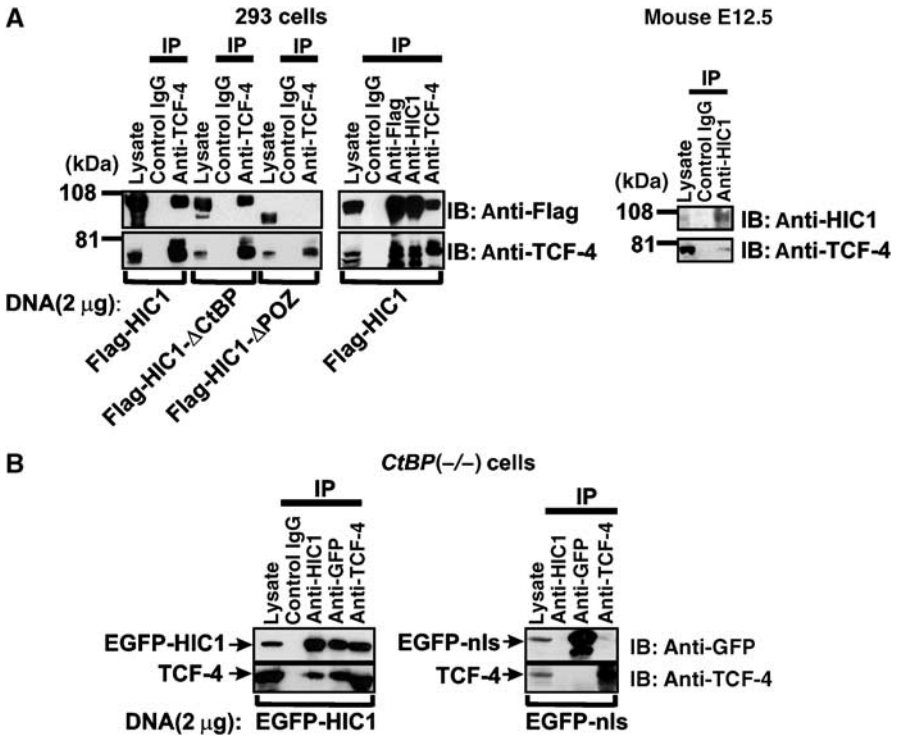

Figure 3 Association between TCF-4 and HIC1 *in vivo*. (**A**) Left, coimmunoprecipitations between endogenous TCF-4 and various exogenous HIC1 proteins in human 293 cells. Right, coimmunoprecipitations of endogenous HIC1 and TCF-4 in cells derived from mouse embryos on day 12.5 p.c. IP, immunoprecipitation; IB, immunoblotting; in lanes denoted 'lysate' five percent of the total sample were loaded. (**B**) TCF-4 associates with HIC1 in *CtBP(−/−)* cells. Coimmunoprecipitation of endogenous TCF-4 with EGFP-HIC1 is specific for HIC1 as evidenced by no detectable interaction between TCF-4 and EGFP-nls in a control experiment (right).

GST-HIC proteins interacted with full-length TCF-4; however, the strongest interacting domain was localized in the internal and C-terminal region of HIC1. Importantly, TCF-4mutCtBP binds to GST-HIC1 proteins equally well as the wild-type protein. Two nonoverlapping TCF-4 fragments were used to delineate domains involved in binding to HIC1. Whereas the C-terminus of TCF-4 interacted strongly with the GST-linked C-terminal part and the HIC1 internal fragments, a substantially less avid interaction was observed between the TCF-4 C-terminus and the GST-HIC1 N-terminus. Finally, the N-terminal part of TCF-4 did not show any affinity to HIC1. TCF-4 and HIC1 bind specific DNA sequences and their interaction domains were mapped in part to the DNA-binding regions of these proteins. To exclude the possibility that the association between TCF-4 and HIC1 is indirect and might be mediated by a DNA bridge from contaminating DNA, we performed a GST-pull-down with translated HIC1-mutZF3 protein containing a single amino acid exchange in the third zinc finger (Figure 4A). This mutation abolishes the DNA binding of the mutated protein to its recognition motif (Pinte *et al*, 2004). We also treated translated TCF-4 and glutathione-Sepharose bound GST-HIC1 with DNase I prior to the pull-down. As shown in Figure 4C, the HIC1-ZF3 associates with GST-TCF-4 with a comparable avidity to the wild-type protein and, moreover, the DNase I treatment even slightly improved *in vitro* binding of TCF-4 and HIC1.

Altogether, the data reported here indicated that HIC1 interacts directly with TCF-4 in a complex multidomain mode of interaction. These data also implied that this interaction is not dependent on the presence of the intact CtBP-binding sites in TCF-4.

HIC1 inhibits TCF/β-catenin-driven transcription

To examine whether HIC1-mediated sequestration of TCF-4 affects the Wnt-dependent transcription, pTOPFLASH was cotransfected with each of the HIC1 constructs into 293 cells, the cells were subsequently stimulated by the Wnt ligand and the levels of the TCF-mediated transcription were determined. In 293 cells, Wnt3a-containing medium induced robust 25-five fold activation of the Tcf reporter pTOPFLASH as compared to the control medium (Figure 5). The cotransfection of wild-type HIC1 resulted in a substantial decrease of the pTOPFLASH activity, the HIC1-ΔCtBP was less than half as effective as wild-type HIC1, and finally, the HIC1-ΔPOZ mutant appeared to be completely inefficient in the downregulation of the pTOPFLASH-driven transcription. We did not observe any effect whatsoever on the pTOPFLASH reporter in the nonstimulated cells. Nevertheless, HIC1 repressed the Wnt-induced transcription of a luciferase reporter containing a 5 kb promoter region of the well-established Wnt target *Axin2* gene (Figure 5) (Jho *et al*, 2002). Conversely, the HIC1 overexpression did not disturb the transcription from the negative-control reporter pFOPFLASH, or from the synthetic reporter G1E1B-Luc activated by Gal4-DBD-VP16 fusion protein (data not shown).

HIC1 contains five Krüppel-like C_2H_2 zinc fingers in its C-terminal part. Recently, Pinte *et al* (2004) investigated the DNA binding properties of the isolated zinc finger domain and defined a specific DNA motif recognized by HIC1. Full-length HIC1 binds probes with a single recognition site poorly; however, the wild-type protein interacts cooperatively with complex probes containing multiple HIC1-specific

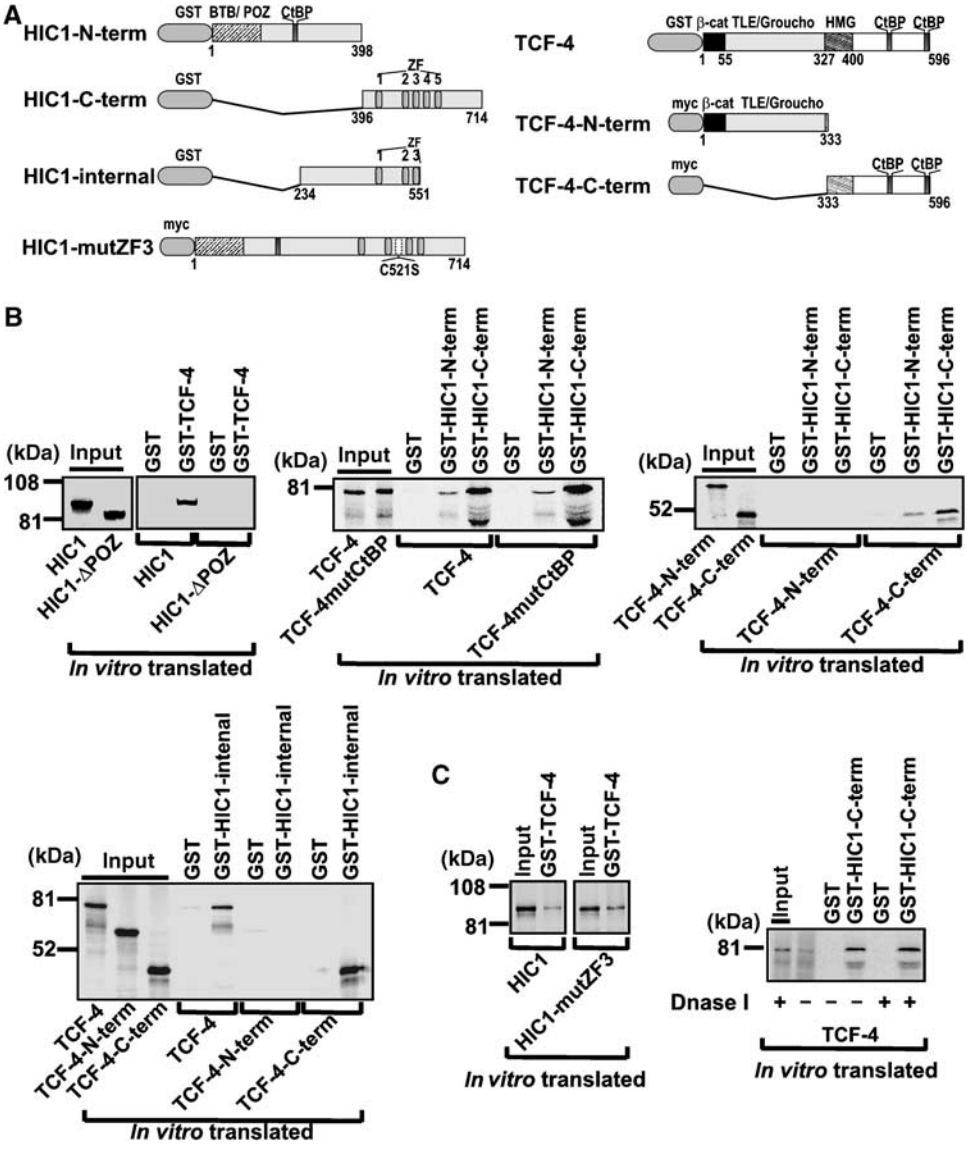

Figure 4 *In vitro* interaction of TCF-4 and HIC1. (**A**) Structures of HIC1 and TCF-4 proteins used in the *in vitro* pull-down assays (see also the diagrams of the additional HIC1 and TCF-4 constructs depicted in Figures 1A and 2A). (**B**) Pull-down assays between bacterially expressed GST-fusion and *in vitro* translated proteins, as indicated. Ten percent of the total reactions were loaded in lanes denoted 'input'. (**C**) Right, TCF-4/HIC1 interaction is resistant to DNase I treatment; left, the intact DNA-binding domain of HIC1 is not essential for the interaction with TCF-4.

sequences. In contrast, the N-terminally truncated HIC1-ΔPOZ, lacking the BTB/POZ domain, interacts preferentially with a probe containing a single HIC1-binding site. We performed an electrophoretic mobility-shift assay (EMSA) with *in vitro* translated TCF-4 and HIC1. HIC1-ΔPOZ, and with a lower efficiency, full-length HIC1, bound to a simple HIC1-specific probe, but these proteins did not interact directly with probes containing single or multiple TCF recognition motifs (Supplementary Figure 1).

The inhibitory effect of HIC1 on the transcriptional activation of pTOPFLASH was also found in DLD-1 cells, that is, cells with a mutant *APC* gene. The lack of functional APC protein in these adenocarcinoma cells results in the accumulation of β-catenin and in constitutive activation of the TCF-dependent target genes (van de Wetering *et al*, 2002; Rosin-Arbesfeld *et al*, 2003). By retroviral transduction, we generated DLD/HIC1 cells containing the *HIC1-EGFP* regulated by AP21967, a synthetic dimerizer (Ariad). We examined the levels of the TCF/β-catenin-dependent transcription simultaneously in three independent DLD/HIC1 cell lines at three different levels of HIC1-EGFP expression (dimerizer concentrations: 0, 0.25 and 25 nM). The result of a representative experiment is shown in Figure 6A. At maximum induction when HIC1 was produced in amounts comparable to the physiological levels of endogenous HIC1 in primary human WI38 cells (Figure 9C), the pTOPFLASH activity decreased to approximately 40% when compared to the DLD/HIC1 cells growing without the inducer (Figure 6A). Transcription from the negative control reporter pFOPFLASH did not change during the experiment. Since DLD-1 cells express high amounts of both TCF-4 and β-catenin (Korinek *et al*, 1997), we asked first whether HIC1 can function by decreasing the intracellular levels of β-catenin and/or TCF-4. Using anti-TCF-4 and β-catenin antibodies, we performed immunoblotting of cell lysates prepared from DLD/HIC1 growing with dimerizer for 5 days or without HIC1 induction.

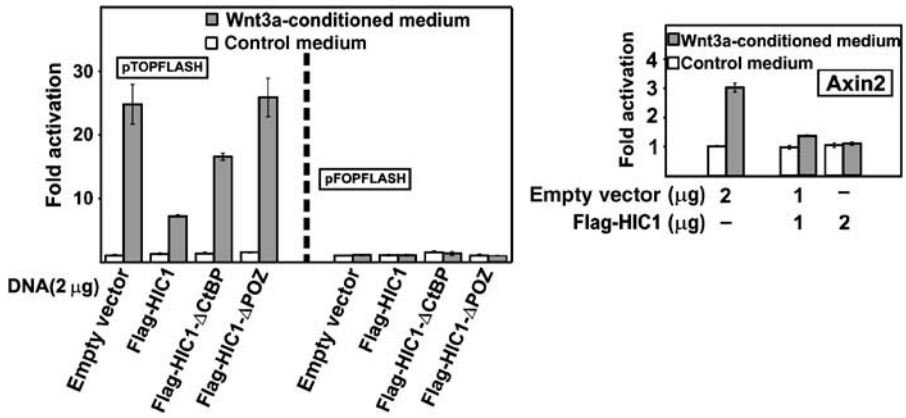

Figure 5 HIC1 represses the Wnt-stimulated transcription. Reporter gene assay with the Wnt-responsive promoters. 293 cells were cotransfected with the reporters and the HIC1 constructs as indicated and stimulated for 24 h with Wnt3a-conditioned or control medium. Luciferase (firefly) activities were corrected for the efficiency of transfection using the internal control *Renilla* luciferase expression plasmid. The reporter activity in unstimulated mock-transfected cells was arbitrarily set to 1. The histograms represent mean values of triplicate experiments and SDs (standard deviations) are shown by error bars.

The analysis revealed that HIC1 overexpression does not reduce the overall levels of the endogenous TCF-4 and β-catenin proteins in the cells (Figure 6A). However, HIC1 sequestered endogenous β-catenin and TCF-4 into the nuclear HIC1 bodies (Figure 6B and Supplementary Figure 2). Using anti-GFP antibody, we coimmunoprecipitated β-catenin, HIC1 and TCF-4 in one complex from 293 cells (Figure 6C). As we did not observe a direct interaction between β-catenin and HIC1 *in vitro* using pull-down assays (Supplementary Figure 3), we concluded that β-catenin associates with HIC1 indirectly, possibly by binding to TCF-4.

In summary, these data indicated that HIC1 specifically represses TCF-mediated transcription. Intriguingly, the repression is dependent on the recruitment of the TCF-4 to the HIC1 bodies rather than on the direct interaction of HIC1 with the promoters of the repressed genes.

HIC1 regulates Axin2 transcription

The *HIC1* gene is silenced by promoter methylation in most of the tumor-derived cell lines tested so far; nevertheless, we detected *HIC1* mRNA expression in human medulloblastoma DAOY cells, human primary fibroblast WI38 cells and also in mouse embryo STO cells. Using anti-HIC1 antibody, we visualized the nuclear HIC1 bodies in all these cells (see higher magnification insets in Figures 7B and 8A; staining of STO cells is not shown). Treatment of DAOY cells with 5-aza-2′-deoxycytidine (5-aza-2′-dCyt), that is, with an agent blocking DNA methylation, resulted in two-fold increase in the levels of *HIC1* mRNA (Figure 7A). This increased *HIC1* expression was also detected at the protein level and the 5-aza-2′-dCyt-treated cells contained a higher amount of larger HIC1 bodies than untreated cells (Figure 7B, insets). We further tested the effect of HIC1 knockdown on Wnt signaling. We found that in WI38 cells the *HIC1* mRNA level was reduced to 20% upon transfection with HIC1 short inhibitory RNAs (siRNAs), compared to an irrelevant control (a mixture of anti-GFP and anti-luciferase siRNAs) (Figure 8A). The effective downregulation of HIC1 in the transfected cells was confirmed by Western blotting and confocal microscopy (Figure 8A, inset). Recently, it was shown by Chen *et al* (2005) that HIC1 binds the SIRT1 promoter and directly represses its transcription. Thus, as expected, upon HIC1 knockdown we observed increased levels of the *SIRT1* mRNA; we also noted a 60% increase of basal transcription of *Axin2*, the Wnt signaling pathway target gene (Figure 8A). Interestingly, upon Wnt stimulation, the HIC1 siRNAs remarkably (almost two-fold) elevated only the transcriptional response of the Axin2 promoter, while the expression of the *SIRT1* and two housekeeping genes remained unchanged (Figure 8B). Such robust activation of *Axin2* was quite astonishing as the HIC1 siRNAs treatment already increased the levels of the *Axin2* mRNA in unstimulated cells. These data, obtained with physiological amounts of HIC1 and with the endogenous Wnt signaling target, supported our observations about HIC1 antagonizing Wnt signaling.

HIC1 diverts TCF-4 and β-catenin from the Wnt-responsive promoters

The mechanism of the HIC1 action on the endogenous TCF-responsive promoters was first studied by ectopic expression of full-length HIC1 in 293 cells. At the mRNA level, HIC1 overexpression partly blocked the increase in abundance of three Wnt signaling responsive genes *Axin2*, *Sp5* and *Cyclin D1* (Shtutman *et al*, 1999; Leung *et al*, 2002; Weidinger *et al*, 2005) observed in control cells upon 12- or 24-h stimulation with Wnt3a (Figure 9A). The inhibition appeared to be incomplete because about 50% transfection efficiency was routinely achieved for the 293 cells (data not shown). Thus, although HIC1 obviously inhibited the *Axin2, Sp5* or *CyclinD1* stimulation in the transfected cells, the overall amounts of the corresponding mRNAs were moderately elevated in Wnt3a-treated cell cultures. The mRNA abundance of *GAPDH* and *CtBP2*, that is genes with no direct relationship to Wnt signaling, did not change in the experiment.

To clarify the mechanisms of the HIC1-mediated repression, ChIP was performed on a cluster of TCF-binding sites in the Sp5 promoter (Supplementary Figure 4) (Takahashi *et al*, 2005). The ChIP assay showed that HIC1 did not associate with the Sp5 promoter; furthermore, the binding of endogenous TCF-4 to this promoter was decreased by wild-type HIC1 overexpression and not by HIC1-ΔPOZ in both stimulated and nonstimulated 293 cells (Figure 9B, only data for Wnt3a-

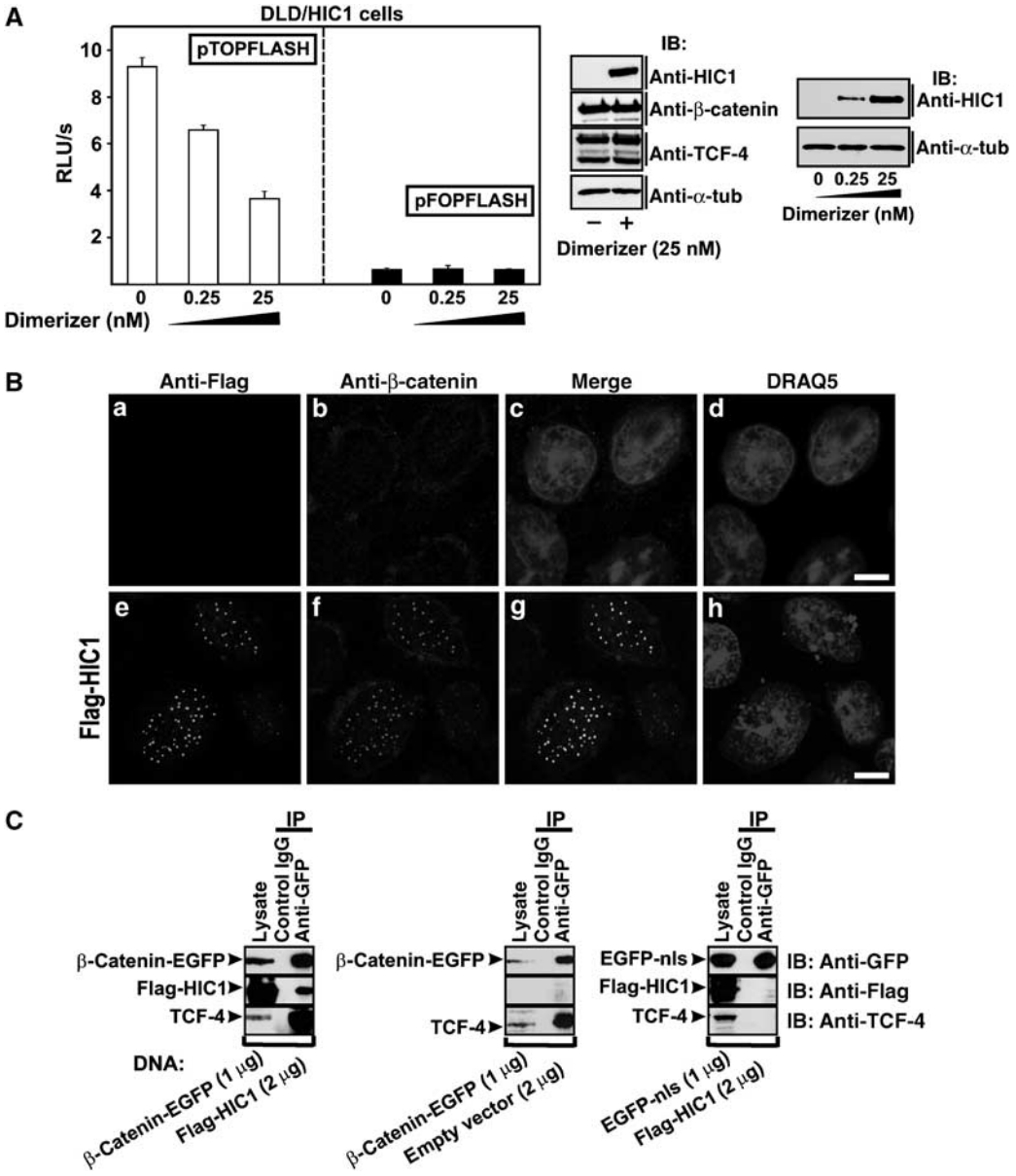

Figure 6 HIC1 represses TCF/β-catenin signaling in DLD-1 adenocarcinoma cells. (**A**) Right, transgenic DLD/HIC1 cells growing at higher concentrations of a synthetic compound AP21967 (dimerizer) contain increasing amounts of the HIC1 protein as evidenced by Western blots of total cell extracts probed with anti-HIC1 antibody. Middle, HIC1 expression does not influence the protein levels of TCF-4 and β-catenin. Left, the constitutive activity of the TCF-dependent reporter pTOPFLASH is suppressed by increasing amounts of the HIC1 protein. Average luciferase light units per second (RLU/s) corrected for the efficiency of transfection determined as the luciferase/*Renilla* ratio from five experiments are given (right, pFOPFLASH values). (**B**) Colocalization of HIC1 with endogenous β-catenin. Confocal micrographs of DLD-1 cells transfected with the full-length Flag-HIC1 construct stained with anti-Flag and anti-β-catenin antibody. Bar, 10 μm. (**C**) HIC1 expression does not disrupt the binding between TCF-4 and β-catenin. Coimmunoprecipitation of endogenous TCF-4 with ectopically expressed β-catenin is not affected by co-expression of HIC1 (compare left and middle panel). The coimmunoprecipitation is specific for β-catenin as indicated by a control experiment using the EGFP-nls instead of β-catenin-EGFP fusion protein (right).

induced cells are shown). We then co-transfected HIC1 and β-catenin, and performed ChIP with an antibody directed against this Wnt effector protein. The results unambiguously showed the presence of β-catenin on the Sp5 promoter in control cells (transfected with an empty vector or with the HIC1-ΔPOZ mutant which is deficient in TCF binding). In contrast, wild-type HIC1 completely eliminated association of exogenous β-catenin with the TCF-responsive element of the *Sp5* gene (Figure 9B).

In DLD-1 cells, we did not detect any *Sp5* mRNA and, moreover, expression of *Axin2* and *Cyclin D1* seemed to be independent of the TCF/β-catenin as the transient transfection of a dominant-negative form of TCF-4 (ΔN-TCF-4) did not reduce transcription of these Wnt signaling target genes. However, ΔN-TCF-4 inhibited production of *Tenascin C*, a recently identified TCF/β-catenin target gene active in colon carcinoma cells growing at the invasive front of the tumors (data not shown) (Beiter *et al*, 2005). For the ChIP assay we used DLD/HIC1 cells with regulated expression of the HIC1-EGFP transgene. Predictably, HIC1 expression did not inhibit the activity of the in DLD-1 cells TCF-independent Axin2 and Cyclin D1 promoters (Figure 9C). This was not caused by

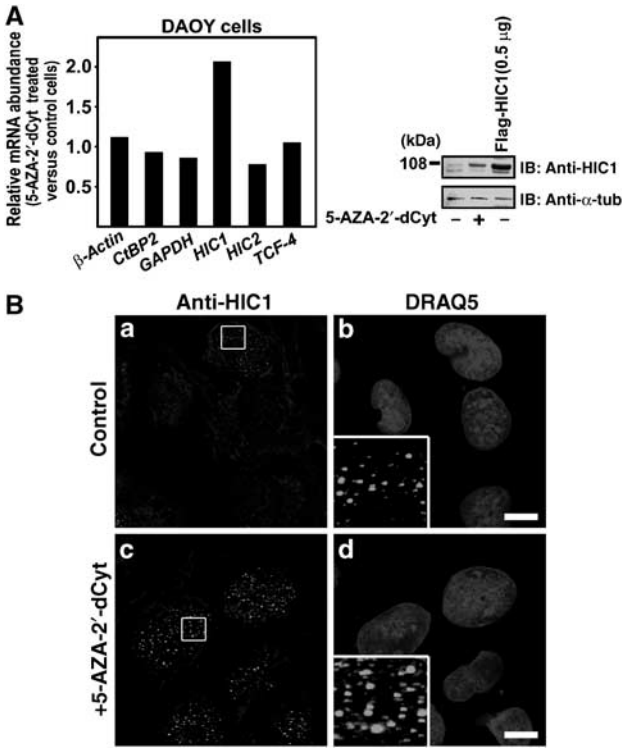

Figure 7 The nuclear HIC1 bodies in DAOY cells. (**A**) Increased expression of *HIC1* mRNA and protein in the cell line DAOY after treatment with 1 μM 5-aza-2′-deoxycytidine for 6 days. The expression was analyzed by qRT–PCR (left) or by Western blotting using anti-HIC1 antibody. In lane 3, a lysate from 293 cells transfected with 0.5 μg of the Flag-HIC1 construct was loaded. (**B**) Confocal micrographs of DAOY cells treated with 5-aza-2′-deoxycytidine (c, d) or with a vehicle (a, b) stained with the affinity purified anti-HIC1 antibody. Bar, 10 μm.

a dysfunction of the HIC1-EGFP fusion protein as HIC1-EGFP efficiently attenuated pTOPFLASH transcription (Figure 6A) and the ectopically expressed HIC1-EGFP construct displayed the same activity as wild-type HIC1 in suppressing. Nevertheless, HIC1 efficiently blocked transcription from a promoter of the *Tenascin C* gene (Figure 9C). ChIP analysis of the proximal TCF-dependent DNA element in the *Tenascin C* promoter (Supplementary Figure 4) revealed a clear binding of TCF-4 and β-catenin in parental DLD-1 cells. In DLD/HIC1, upon induction with the dimerizer HIC1 significantly decreased the association of both TCF-4 and β-catenin with the *Tenascin C* promoter, but no interaction of this promoter, with HIC1 was observed (Figure 9D). Simultaneously, the functionality of HIC1-EGFP to bind its cognate DNA motif was confirmed by ChIP of the HIC1-recognition element in the SIRT1 promoter (Figure 9D). These data indicate that as a result of the recruitment to the HIC1 bodies, TCF-4 and β-catenin are prevented from associating with TCF target genes.

Discussion

Since the canonical Wnt/β-catenin signaling alone is unlikely to regulate multiple developmental programs initiated by the Wnt ligands, we searched for additional proteins that could modulate the function of the TCF/LEF factors. In the present study, we identified tumor suppressor HIC1 as a new nuclear modulator of the Wnt signaling pathway.

Nuclear sequestration of TCF-4 into the HIC1 bodies

HIC1 encodes a zinc-finger transcription factor that acts as a transcriptional repressor. Additionally, the HIC1 protein binds CtBP and via its N-terminal BTB/POZ domain forms nuclear aggregates known as HIC1 bodies. Here, we present convincing data based on confocal microscopy showing the colocalization of the Wnt signaling effector protein, TCF-4, HIC1 and CtBP1 in these nuclear bodies. We further show that HIC1 directly associates with TCF-4 *in vitro* and *in vivo*. However, the efficient sequestration of TCF-4 into the HIC1 bodies depends on the presence of the CtBP in the TCF-4/HIC1 complex. Strikingly, in CtBP-positive cells, wild-type HIC1 and the TCF-4mutCtBP protein (this variant lacks the CtBP-binding motifs but is still able to interact with HIC1 *in vitro* (Figure 4A and B)) do not display an overlapping localization (Figure 2C). We speculate that CtBP induces a specific spatial arrangement of the HIC1 bodies. Such arrangement possibly favors the recruitment of TCF-4 via the interaction with CtBP. This conclusion is supported by a different morphology of the HIC1 aggregates, which in the presence of CtBP show more compact appearance than the HIC1/TCF-4 bodies without CtBP (Figure 2C). The absence of HIC1 and TCF-4mutCtBP colocalization argues against the obvious objection that overexpressed proteins aggregate and sequester other proteins that they do not normally interact with.

Using *in vitro* pull-down assays, we mapped the main regions of the interaction to the C-terminal parts of HIC1 and TCF-4. Surprisingly, whereas GST-HIC C-terminal and GST-HIC internal fragments interacted with *in vitro* translated TCF-4, the N-terminally truncated HIC-ΔPOZ variant failed to interact with full-length GST-TCF-4. We assume that the anchoring of the HIC1 protein fragments to GST-beads possibly prevents the incorrect folding induced by the deletion of the structurally essential BTB/POZ domain.

Many nuclear factors involved in pre-mRNA splicing, regulation of transcription, apoptosis or cell cycle progression are localized in distinct structures called speckles or nuclear bodies (reviewed in Lamond and Spector, 2003). Using confocal microscopy we visualized HIC1 bodies at endogenous expression levels in the nuclei of three different cell types. Is the physical sequestration of the transcription factors main function of the HIC1 bodies or are there some other physiological roles for these structures? What is the proportion between the 'free' HIC1 protein (i.e. HIC1 associated with promoters or other factors) and HIC1 aggregated in the bodies? More experimental work needs to be carried out to answer these questions.

HIC1 represses TCF-mediated transcription

The results presented here show that full-length HIC1 substantially reduced the levels of TCF-mediated transcription of two different TCF/β-catenin-regulated reporters (Figure 5), and furthermore, several selected endogenous Wnt signaling target genes were also affected by HIC1 expression (Figures 8 and 9). Importantly, HIC1 knockdown in normal cells enhanced the levels of the transcriptional stimulation induced by the Wnt3a ligand (Figure 8B). Thus, HIC1 specifically repressed transcription dependent on TCF and this repression occurred at physiological levels of the HIC1 protein. Interestingly, we observed that the activator role of Wnt signaling was dependent on the particular cellular back-

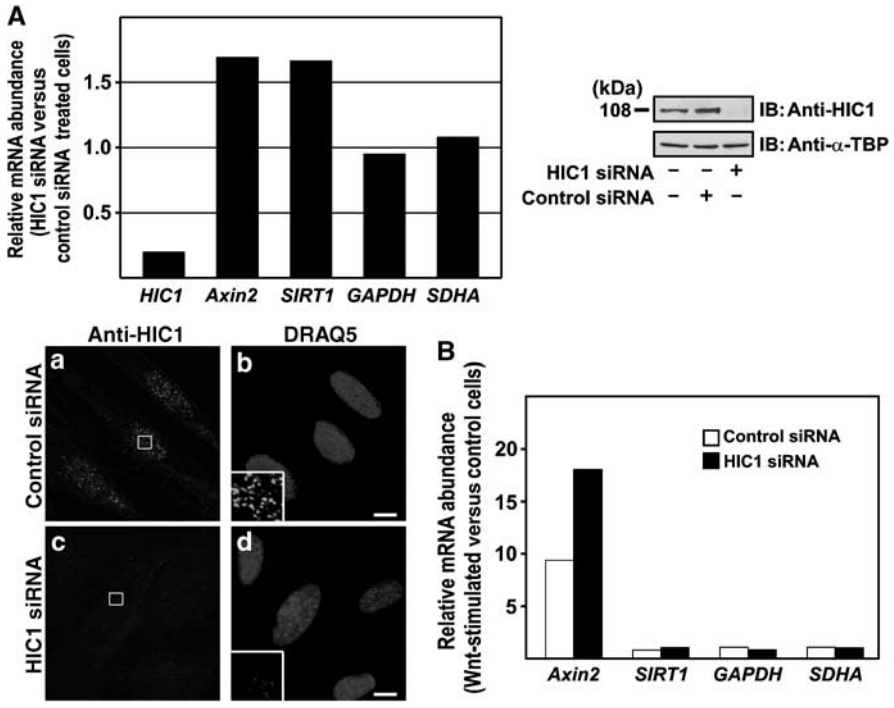

Figure 8 HIC1 knockdown increases the TCF-mediated transcription. (**A**) Human primary cells WI38 were transfected with HIC1 siRNAs or control siRNAs and the changes in the levels of *HIC1* mRNA or protein were tested 24 h post-transfection. Left, results of the qRT–PCR analysis. The relative abundance of the given mRNA in HIC1 siRNA versus control siRNA-transfected cells was derived from the average CT values of four independent experiments after normalizing to the levels of β-*actin* cDNA. Right, Western blots of nuclear extracts prepared from the indicated cells. Bottom, confocal micrographs of WI38 cells transfected with the indicated siRNAs and stained with the affinity purified anti-HIC1 antibody. Bar, 10 μm. (**B**) The activity of the Wnt-dependent promoter of the *Axin2* gene is increased by HIC1 knockdown. Results of qRT–PCR analysis performed with cDNA generated from WI38 cells transfected with the indicated siRNAs upon 24-h stimulation with Wnt3a. Six PCR reactions were done for each primer set. The relative abundance of the indicated mRNA in Wnt3a-stimulated versus control cells was derived from the average CT values after normalizing to the levels of β-*actin* cDNA.

ground. For example, the stimulation by Wnt3a activated transcription of *Sp5*, *Axin2* and *Cyclin D1* in 293; in primary fibroblast WI38 cells, only robust transactivation of the *Axin2* gene was detected, while the expression levels of *Sp5* and *Cyclin D1* remained under detection limits. In colon carcinoma DLD-1 cells, that is, in cells with constitutive active Wnt signaling, ectopic expression of a dominant-negative (blocking) form of TCF-4 inhibited expression of *Tenascin C* but not *Axin2* and *Cyclin D1* mRNA. This clearly indicates that gene expression is in general regulated by inputs from various cellular pathways that integrate in the regulatory regions of a particular gene. Nevertheless, HIC1 regulated the transcriptional response of all tested genes showing the reactivity to the Wnt signal in the given cell type.

Mechanisms of HIC1-mediated inhibition

It was well documented that Kaiso, a member of the BTB/POZ protein family, interacts with sequence-specific elements in several Wnt target genes (Park *et al*, 2005). In *Xenopus*, Kaiso and TCF act in concert on the *siamois* promoter. The Kaiso's general role is not completely understood, but it is likely to also include a direct binding and the recruitment of corepressors such as N-CoR to a subset of Wnt targets. Recently, Chen *et al* (2005) showed that HIC1 forms a transcriptional repression complex with SIRT1 deacetylase. This complex directly binds and represses transcription from the promoter of the *SIRT1* deacetylase gene.

ChIP analysis revealed that HIC1 does not bind directly or indirectly (i.e. via TCF-4) the regulatory elements in the TCF-responsive genes. In contrast, HIC1 partly reduces the occupancy of the promoter of the *Sp5* gene by endogenous TCF-4. This partial sequestration was expected as only a fraction of the cells expressed exogenous HIC1. In addition, HIC1 eliminated binding of ectopically expressed β-catenin to the *Sp5* promoter.

ChIP analysis of the TCF-binding element in the *Tenascin C* promoter in DLD/HIC1 cells showed a substantial decrease in association of TCF-4 and β-catenin upon HIC1 induction. Taken together, these data indicate that HIC1-mediated sequestration prevents TCF-4 from binding its target promoter. Although β-catenin targeting to the HIC1 bodies seems to be indirect and mediated via its interaction with TCF-4, we cannot completely exclude participation of an unknown factor involved in relocation of β-catenin into the HIC1 bodies (Figure 10). These results imply that HIC1-mediated sequestration can uncouple the TCF/β-catenin-regulated promoters from various inputs related to Wnt signaling, but still may leave such promoters responsive to other regulatory signals.

Materials and methods

Plasmids and RNAi

All constructs were made by standard molecular biology techniques. Triple amino-acid substitutions (Q_{509} to N_{509}, P_{510} to A_{510}, L_{511} to S_{511}, Q_{586} to N_{586}, P_{587} to A_{587}, L_{588} to S_{588}) were introduced into

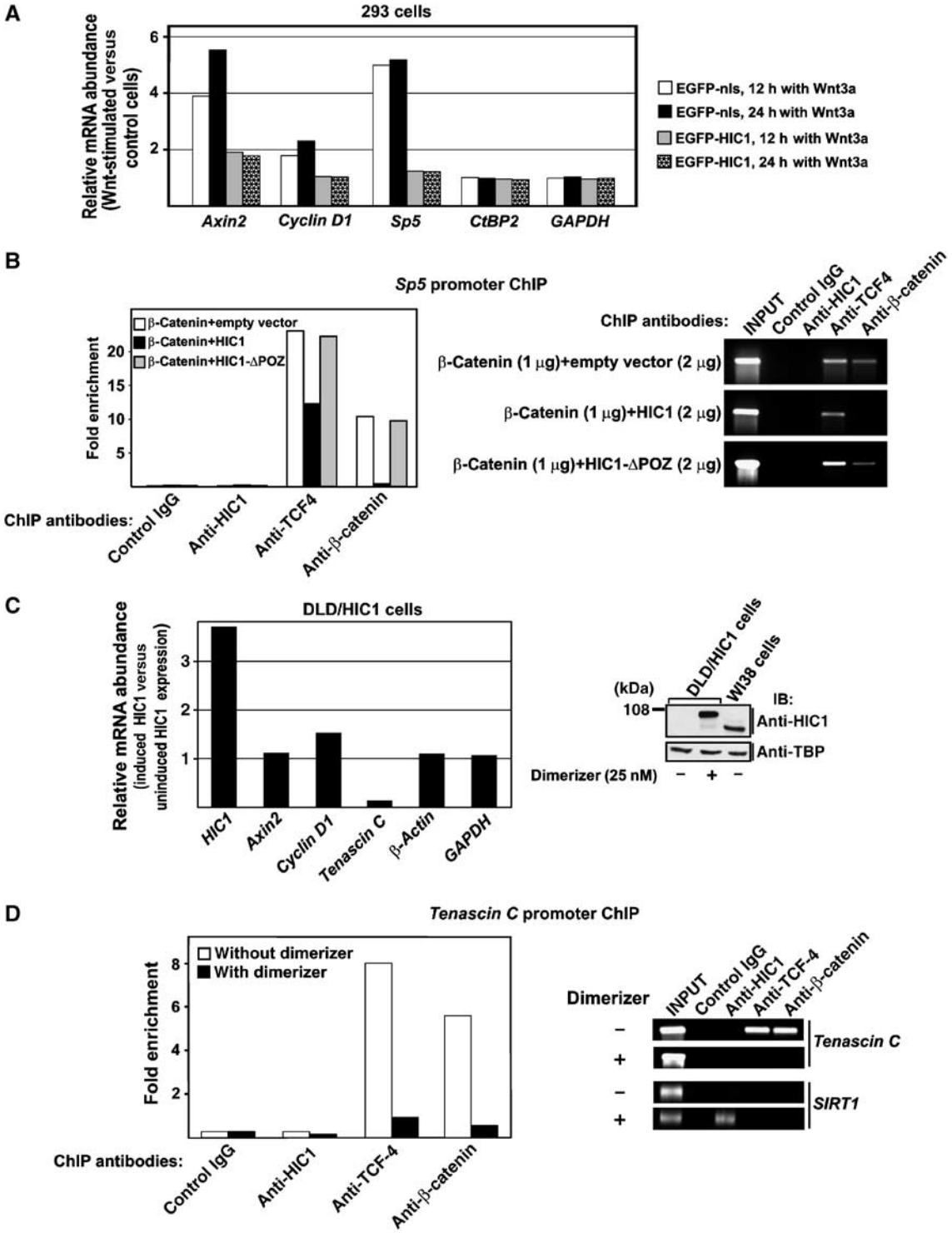

Figure 9 HIC1 sequesters TCF and β-catenin from the TCF-dependent promoters. (**A**) HIC1 blocks transcriptional activation of the Wnt signaling target genes. Results of qRT–PCR analyses performed with cDNA generated from 293 cells transfected with the indicated constructs upon 12- or 24-h stimulation with Wnt3a. Four to six PCR reactions were performed for each primer set. The relative abundance of the indicated mRNA in Wnt3a-stimulated versus control cells was derived from the average CT values after normalizing to the levels of β-*actin* cDNA. (**B**) HIC1 is not associated with the Sp5 promoter but sequesters TCF-4 and β-catenin from the TCF-specific DNA element of this promoter. ChIP analysis of chromatin isolated from 293 cells transfected with the indicated constructs. The diagram at the left represents real-time PCR values obtained with primers spanning the respective DNA element, normalized to the inputs. The image on the right depicts relevant PCR products after 29 cycles of amplification. (**C**) HIC1-EGFP blocks transcription of the Tenascin C promoter in DLD-1 cells. Left, results of qRT–PCR analyses performed with cDNA prepared from DLD/HIC1 cells growing in the presence of the dimerizer (25 nM; HIC1 induction) or without induction. Right, Western blot analysis of nuclear extracts isolated from DLD/HIC1 and WI38 cells. (**D**) HIC1 sequesters TCF-4 and β-catenin from the *Tenascin C* promoter. Left, ChIP analysis of chromatin isolated from DLD/HIC1 cells prior to and upon HIC1-EGFP induction. The diagram at the left represents real-time PCR values obtained with primers spanning the proximal TCF-binding element in the *Tenascin C* promoter, normalized to the inputs. The image on the right depicts relevant PCR products after 29 cycles of amplification. Right bottom, although HIC1-EGFP is not associated with the *Tenascin C* promoter, it binds its recognition element in the SIRT1 promoter.

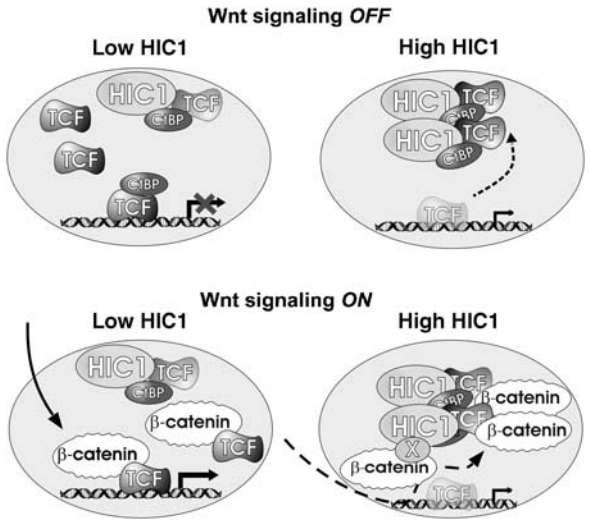

Figure 10 A model for HIC1 suppression of the transcriptional response induced by Wnts. The regulation of a complex promoter integrating inputs from the Wnt and other signaling pathways is depicted. At low levels of HIC1 the activity of the promoter depends mainly on the Wnt signaling components. High levels of HIC1 uncouple the promoter from Wnt signaling. X depicts a hypothetical factor mediating besides TCF-4 the interaction between β-catenin and HIC1.

each of the CtBP-binding sites in TCF-4mutCtBP by using a site-directed mutagenesis kit (Stratagene). For gene knockdowns HIC1 siRNAs were purchased from Ambion. See the Supplementary data for more detailed description of the plasmids used in this study.

Cell culture and transfections

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. All cell lines were regularly checked for the presence of mycoplasma. Transfections were performed using the Lipofectamine reagent (Invitrogen) as described by the manufacturer. DLD/HIC1 cells were produced using ARGENT Regulated Transcription Retrovirus Kit (Ariad). CtBP(−/−) cells derived from the $CtBP1^{-/-}CtBP2^{-/-}$ embryos were kindly provided by J Hildebrand.

Production of Wnt3a-conditioned medium

Mouse Wnt3a-producing L cells (L Wnt-3A; ATCC No. CRL-2647) were cultured in complete DMEM supplemented with G418 (0.5 mg per ml; Alexis). Control and Wnt3a-conditioned medium was prepared according to the protocol provided by the supplier.

Luciferase assays

Reporter gene assays were performed as described previously (Valenta *et al*, 2003). To assay TCF-mediated transcription, firefly luciferase pTOPFLASH and pFOPFLASH (Korinek *et al*, 1997) and the *Axin2* promoter reporter constructs (Jho *et al*, 2002) (a gift from F Costantini) were used. The G1-E1B-Luc reporter and the Gal4-DBD-VP16 construct were kindly provided by C Svensson.

EMSA

Full-length TCF-4, full-length human HIC1 and HIC1-ΔPOZ protein were produced *in vitro* using the Quick TNT Coupled Reticulocyte System (Promega). The assay was performed as reported previously (Valenta *et al*, 2003).

GST interaction assays and DNase I treatment

GST-TCF-4, GST-β-catenin, GST-HIC1-N-term (aa 1–398), GST-HIC1-C-term (aa 396–714) and GST-HIC1-internal (aa 234–551) fusion proteins were expressed in the BL21 (DE3) strain of *Escherichia coli* using the pET-42b vector (Novagen). Relevant proteins were produced *in vitro* using the Quick TNT Coupled Reticulocyte System (Promega). The detailed protocol for the GST pull-downs was described previously (Valenta *et al*, 2003). DNase I treatment of GST-bound or *in vitro* translated proteins was performed in 1 × DNase I buffer (Invitrogen), for 30 min at RT with 0.2 U of DNase I (Invitrogen) per 1 μl of the reaction mixture.

Antibodies

Antisera to TCF-4, HIC1, CtBP1, β-catenin and EGFP were produced by immunization of rabbits with bacterially expressed proteins; mouse mAbs to TCF-4 and HIC1 were prepared using standard techniques from splenocytes of mice immunized with a bacterially produced TCF-4 fragment (aa 31–333) and HIC1 fragment (aa 230–404) respectively. The following commercially available mouse monoclonal antibodies were used: anti-β-catenin (Santa Cruz Biotechnology), anti-dephospho-β-catenin (Alexis), anti-CtBP (Santa Cruz Biotechnology), anti-GFP (BD Clontech), anti-Myc 9E10 (Roche Molecular Biochemicals), anti-Flag M2 (Sigma), anti-Flag (Exbio Praha).

Immunofluorescent microscopy

Cells grown on coverslips were fixed 24 h after transfection in cold methanol (−20°C, 5 min) and then briefly in acetone (−20°C). Fluorochromes were ALEXA 488, 594, and 680 dyes (dilution 1:500; Invitrogen), Cy5 dye (1:500, Amersham Pharmacia Biotech). The samples were mounted in MOWIOL (Calbiochem) containing nuclear staining dye DRAQ 5 (1:750; Alexis). Immunofluorescences were visualized using a confocal laser scanning microscope (TCS SP; Leica). All images were scanned separately in the 'sequential scanning mode' for the green, red and blue channels using a × 100/1.40 oil-immersion objective. The ratio of colocalization was quantified by measuring the overlap in the fluorescence intensities of corresponding channels along selected profiles using Leica confocal software. Image files were processed with Adobe Photoshop.

RNA purification and real-time qRT–PCR

Standard procedures were used for RNA purification and reverse transcription. The primers used are listed in Supplementary Table S1 (Supplementary data). The cycling was performed in an Mxp3000 instrument (Stratagene).

ChIP

293 cells stimulated with Wnt3a-conditioned or control medium, DLD-1 and DLD/HIC1 cells were subjected to the ChIP assays according to Kirmizis *et al* (2004). See the Supplementary data for the primer sequences.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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Supplementary information

Materials and Methods

Plasmids and mutagenesis

Full-length HIC1, HIC1-ΔCtBP (deleted aa 222-228) and HIC1-ΔPOZ (aa 136-714) constructs encoding proteins of human origin containing N-terminal or C-terminal EGFP were generated by standard cloning procedures in pEGFP-C1 and pEGFP-N3 vector (BD Clontech), respectively. Constructs encoding HIC1 with an N-terminal Flag-tag (in pcDNA3) (Deltour et al., 2002) were provided by D. Leprince. Plasmids encoding HIC1 tagged with a C-terminal Flag were generated by subcloning into pCMV-Flag-5a vector (Sigma). Myc- and EGFP-tagged constructs encoding full-length mouse or human CtBP1 and human TCF-4 were described previously (Valenta et al., 2003). Plasmids encoding the N-terminal (TCF-4-N-term; aa 1-333) or C-terminal fragment of human TCF-4 (TCF-4-C-term; aa 333-596) were prepared in the pK-myc vector (Valenta et al., 2003) by standard cloning techniques. For retroviral transduction, EGFP-tagged human HIC1 was ligated into the pLHIT variant of pLNIT vector (provided by F.H.Gage); untagged mouse CtBP1 was expressed from pBABE-puro retroviral plasmid (a gift from J. Hildebrand). Details of plasmids and sequences of primers used for the mutagenesis are available on request.

Primers for ChIP

The primers used for the PCR amplification were: *Sp5* promoter, sense: 5'-TCCAGACCAACAAACACACC -3' and antisense 5'- GCTTCAGGATCACCTCCAAG -3'; *Tenascin C* promoter, sense: 5'-ACTGGGGCTGGAACAAAGAT-3' and antisense 5'-AGCGAGTACAGGGACTGAGC-3'; *SIRT1* promoter, sense: 5'- GATAGAAACG CTGTGCTCCA-3' and antisense 5'- CCTTCCTTTCTAGCGTGAGC-3'; negative control regions were derived from the distal exon of the *Axin2* gene, sense: 5'-CTGGCTTTGGTGAACTGTTG-3' and antisense 5'- AGTTGCTCACAGCCAAGACA-3', or from the distal exon of the *Tenascin C* gene, sense: 5'-CAGTAGAGGCAGCCCAGAAC-3' and antisense 5'- AGAGAGAGGGGTTGTGCTGA-3'.

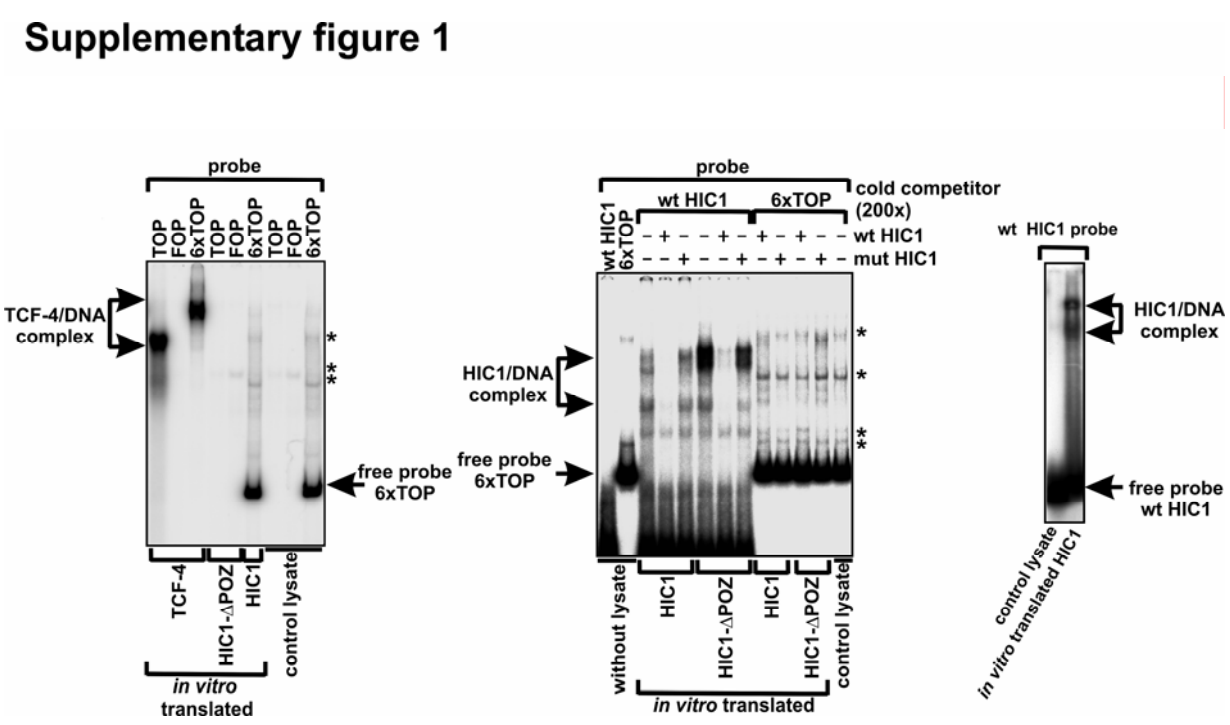
Supplementary Table S1 A set of oligonucleotide primers used for RT-PCR

gene	origin	sequence
Axin1	human	forward 5'-CCTGTGGTCTACCCGTGTCT-3'
		reverse 5'-GCTATGAGGAGTGGTCCAGG-3'
	mouse	forward 5'-ACCCAGTACCACAGAGGACG-3'
		reverse 5'-CTGCTTCCTCAACCCAGAAG-3'
Axin2	human	forward 5'-CTGGCTTTGGTGAACTGTTG-3'
		reverse 5'-AGTTGCTCACAGCCAAGACA-3'
	mouse	forward 5'-TAGGCGGAATGAAGATGGAC-3'
		reverse 5'-CTGGTCACCCAACAAGGAGT-3'
beta-actin	human	forward 5'-GGCATCCTCACCCTGAAGTA-3'
		reverse 5'-AGGTGTGGTGCCAGATTTTC-3'
	mouse	forward 5'-GATCTGGCACCACACCTTCT-3'
		reverse 5'-GGGGTGTTGAAGGTCTCAAA-3
CtBP2	human	forward 5'-CCATCCAGTGGAGGTTTGTC-3'
		reverse 5'-AGCGTATCGTAGGAGTGGGA-3'
Cyclin D1	human	forward 5'-CCATCCAGTGGAGGTTTGTC-3'
		reverse 5'-AGCGTATCGTAGGAGTGGGA-3'
GAPDH	human	forward 5'-CACCACACTGAATCTCCCCT-3'
		reverse 5-CCCCTCTTCAAGGGGTCTAC-3'
	mouse	forward 5'-AACTTTGGCATTGTGGAAGG-3'
		reverse 5-ATCCACAGTCTTCTGGGTGG-3'
HIC1	human	forward 5'-CGACGACTACAAGAGCAGCA-3'
		reverse 5'-TGCACACGTACAGGTTGTCA-3'
	mouse	forward 5'-CAACCTGTACGTGTGCATCC-3'
		reverse 5'-ACGTGTGCATTCAGCTGTTC-3'
HIC2	human	forward 5'-CTCCACAGTGTTCCAGCAGA-3'
		reverse 5'- CAGCAGCTTGCCTGTGTAGA-3'
SDHA	human	forward 5'-AGATTGGCACCTAGTGGCTG-3'
		reverse 5'-ACAAAGGTAAGTGCCACGCT-3'
	mouse	forward 5'-AAGGCAAATGCTGGAGAAGA-3'
		reverse 5'-TGGTTCTGCATCGACTTCTG-3'
SIRT1	human	forward 5'-GCAGATTAGTAGGCGGCTTG-3'
		reverse 5'-AGCGCCATGGAAAATGTAAC-3'
Sp5	human	forward 5'-ACTTTGCGCAGTACCAGAGC-3'
		reverse 5'-ACGTCTTCCCGTACACCTTG-3'
TCF-4	human	forward 5'-TAGCTTTAGCGTCGTGAACC-3'
		reverse 5'-GGGGGCAAATTAAGAAAAGTG-3'
Tenascin C	human	forward 5'-GTCACCGTGTCAACCTGATG-3'

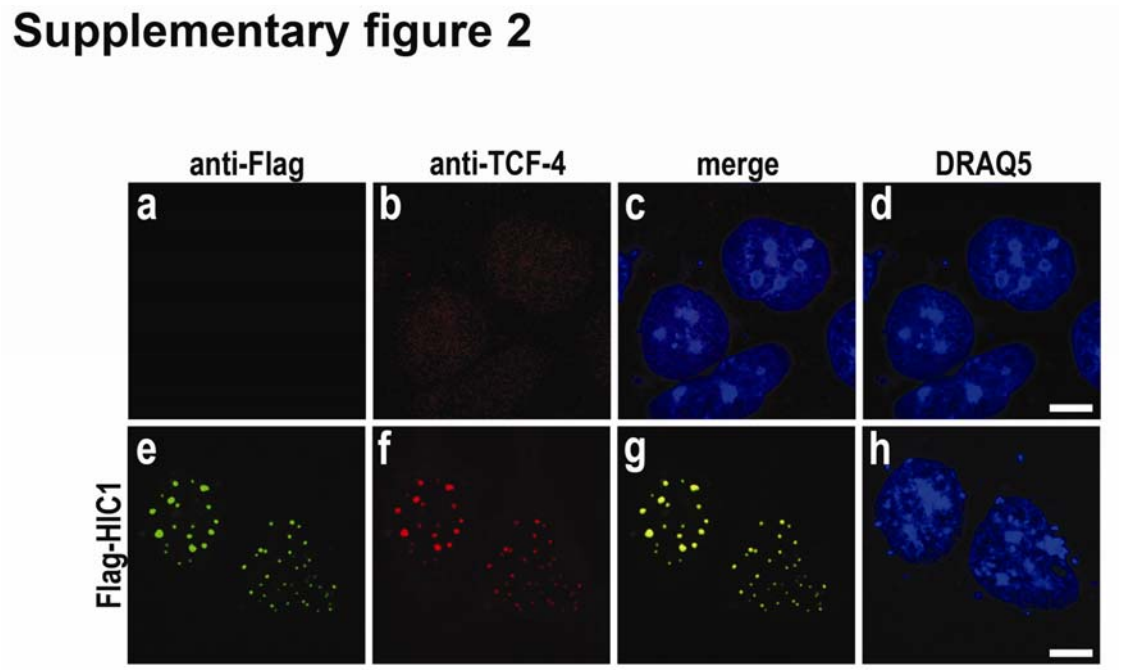
reverse 5'-GTTAACGCCCTGACTGTGGT-3'

Supplementary figures

Supplementary Figure S1 TCF-4 and HIC1 differ in the DNA-binding specificities.
A gel-retardation assay performed with the TCF-4 and HIC1 proteins translated *in vitro*. As the optimal Tcf/Lef probe, a double-stranded 12-nucleotide oligomer 5'-ACCCTTTGATCT-3' was used; the control probe was 5'-ACCCTTTGGCCT-3'. A PCR fragment amplified from pTOPFLASH (Upstate Biotechnology) containing 6 copies of the Tcf/Lef interaction motif was used as the multiple TCF-specific (6xTOP) probe. The wild-type HIC1 probe was double-stranded oligonucleotide 5'-GGGCCTGGGGGCAACCCAATCAC-3', the negative control HIC1mut was 5'-GGGCCTGGGGATGACCCAATCAC-3'. TCF-4/DNA and HIC1/DNA complexes and the positions of the free probes are indicated by arrows. Stars indicate non-specific bands.



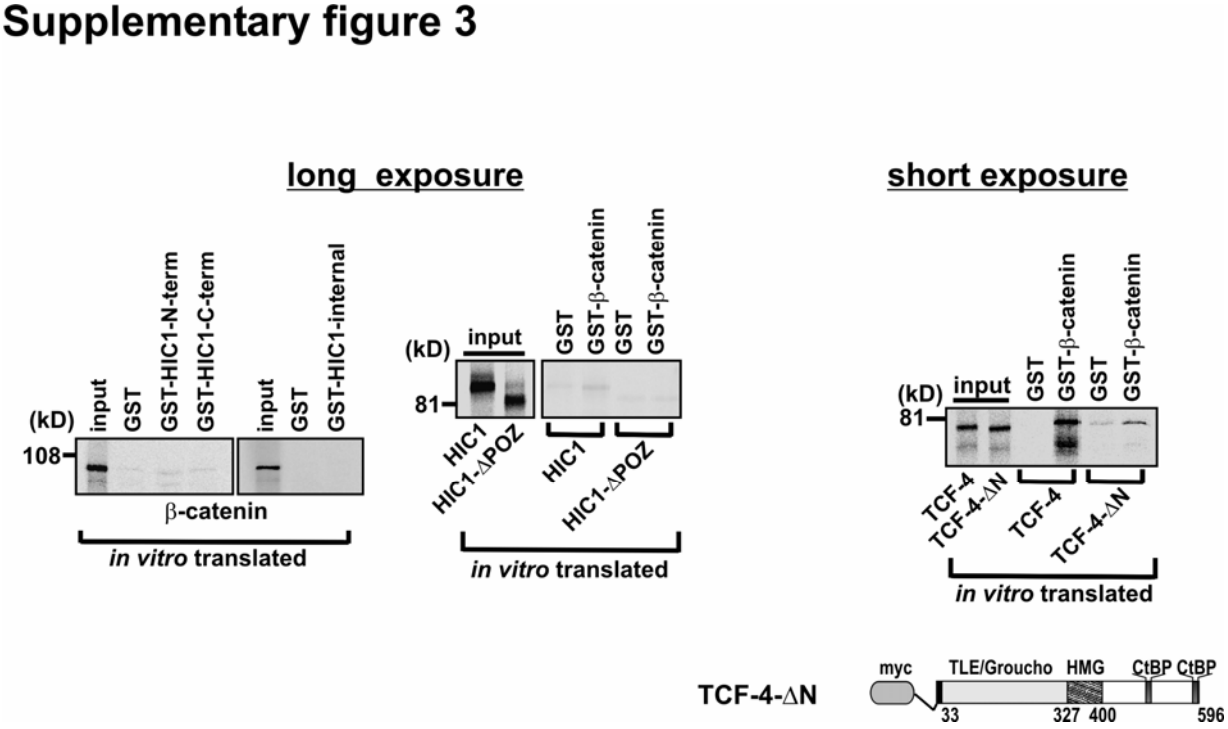
Supplementary Figure S2 HIC1 bodies in DLD-1 adenocarcinoma cells contain endogenous TCF-4. Colocalization of HIC1 with endogenous TCF-4. Confocal micrographs of DLD-1 cells transfected with full-length Flag-HIC1 construct stained with anti-Flag and anti-TCF-4 . Bar, 10μm.



Supplementary Figure S3 HIC1 and β-catenin do not interact directly**.** Pull-down assays between bacterially expressed GST-fusion and *in vitro* translated proteins as indicated. Ten percent of the total reactions were loaded in lanes denoted "input". The gel images marked "long exposure" were obtained after a three-day exposition to the BAS-phosphoimager screen, the image named "short exposure" was gained after a standard overnight exposition. No obvious interaction was detected between β-catenin and HIC1, although the same isolate of GST-β-catenin clearly interacted with full-length TCF-4. A structure of the dominant negative

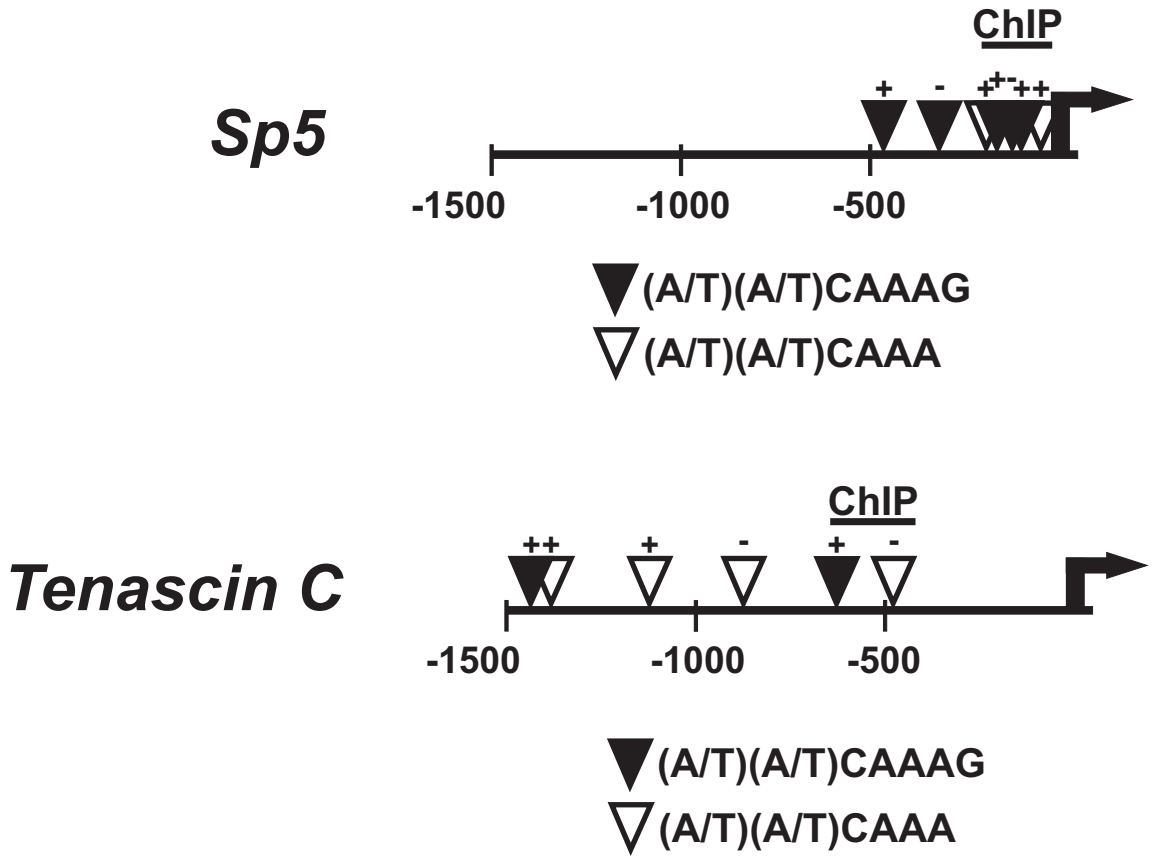
TCF-4-ΔN construct lacking the main β-catenin interaction domain (used as a negative control) is also shown (bottom).

Supplementary figure 3



Supplementary Figure S4 Schematic representation of the human *Sp5* and *Tenascin C* promoters. The TCF-binding sites are depicted by open (basic) or filled (optimal) triangles. The orientation of these sites is indicated by (+) or (-). Areas used for ChIP are shown above the relevant promoter.

Supplementary Figure 4



3.3 Interakce mezi nukleárním receptorem a β-kateninovou signalizací rozhoduje o osudu buněk v somatické gonádě *Caenorhabditis elegans*

Gonáda hlístice *Caenorhabditis elegans* je modelovým příkladem situace, kdy z jedné mateřské buňky vznikají asymetrickým buněčným dělením dvě odlišné buňky dceřiné. Takto vzniklé dceřiné buňky se vzájemně liší přítomností a aktivitou různých molekul, které určují různý vývojový potenciál každé z těchto buněk. Asymetrické buněčné dělení je opakem situace, kdy z mateřské buňky vznikají dvě naprosto identické dceřiné buňky, jejichž rozdílný osud je pak až následně určen různými vnějšími faktory [219, 220]. Gonáda *C. elegans* se vyvíjí ze dvou somatických buněčných prekurzorů (označovaných jako Z1 a Z4), které ohraničují prekurzory zárodečných buněk (Z2 a Z3). Distální dceřiné buňky prekurzorů Z1 a Z4 se po dalším asymetrickém dělení mění v unikátní buňky označované jako DTC (Distal Tip Cell), přičemž jedna DTC je výsledkem několika dělení buňky Z1 a druhá DTC buňka vzniká z buňky Z4. V případě hermafrodita (v populaci výrazně převažující pohlaví) *C. elegans*, je každá z DTC buněk lokalizována na opačném konci gonády a je zodpovědná za růst jednotlivých gonadálních ramen a stimulaci dělení zárodečných buněk. Proximální dceřiné buňky vzniklé z buněk Z1 a Z4 se dále dvakrát dělí a z takto vzniklých buněk dává jedna vznik kotvící buňce (anchor cell) AC. Aktivita AC buňky je nezbytná pro indukci vulvy [221, 222].

Pro určení osudu buňky, a to rovněž v případě *C. elegans* je rozhodující aktivita evolučně konzervovaných signálních kaskád. Jednou z takových klíčových kaskád je signalizace Wnt. Současný model popisující vývoj gonády *C. elegans* vychází ze skutečnosti, že u přímých dceřiných buněk vzniklých z buněk Z1/Z4 je asymetricky distribuován transkripční faktor POP-1, který je jediným zástupcem transkripčních faktorů Lef/Tcf u *C. elegans*. Zvláštností signalizace Wnt u *C. elegans* je existence několika signalizačních β-kateninů (WRM-1, SYS-1 a BAR-1). Při vývoji gonády se uplatňují dva z těchto β-kateninů. V buňce s distálním osudem (buňka DTC) se WRM-1 spolu s kinázou LIT-1 podílí na fosforylaci transkripčního faktoru POP-1. Fosforylovaný POP-1 je exportován ven z buněčného jádra. Zbývající nefosforylováná frakce POP-1 se váže na druhý typ β-kateninu, SYS-1, který je v buňkách exprimován konstantně na relativně nízké úrovni. Heterokomplexy POP-1/SYS-1 aktivují transkripci distálně specifických genů. V proximálních (AC) buňkách k fosforylaci POP-1, a tím k jeho exportu z jádra nedochází. Množství POP-1 v jádře proximálních buněk tak mnohonásobně převyšuje množství SYS-1, a tudíž většina volného proteinu POP-1 působí jako transkripční represor. Ovlivnění

funkce genů *pop-1, sys-1, wrm-1, lit-1* mutací nebo nefyziologickou inaktivací vede k takzvanému Sys (Symetrical sisters) fenotypu, kdy se všechny somatické prekuzorové buňky gonády (dále jen SGP, Somatic Gonad Precursors) mění v buňky s proximálním osudem (AC) a to na úkor buněk distálních (DTC). Signalizace Wnt v tomto případě tedy funguje jako rozhodující faktor pro určení distálního osudu SGP. [106, 107, 223, 224].

Spolu s kolegy z Biologického centra v Českých Budějovicích jsme si položili otázku, zda existuje nějaký faktor, jenž by naopak determinoval proximální osud somatických prekurzorů gonády. Jak se ukázalo takovým faktorem je protein NHR-25 (Nuclear Hormone Receptor 25), transkripční faktor ze skupiny jaderných receptorů pro hormony. NHR-25 je v gonádě exprimován v prekurzorových buňkách Z1 a Z4, jak na úrovni mRNA, tak proteinu. Pokud je u *C. elegans* NHR-25 nefyziologicky inaktivován objevují se v jeho gonádě nadbytečné distální buňky, a to na úkor buněk proximálních. Jedinci s takto inaktivovaných genem nemají funkční gonádu a jsou sterilní. Tento fenotyp je přesně komplementární k Sys fenotypu, tedy k fenotypu jedinců, u nichž došlo k mutaci nebo inaktivaci genů pro komponenty signalizace Wnt. Výsledkem Sys fenotypu je také nefunkční gonáda a reprodukční sterilita. Pokud ovšem nefyziologicky inaktivujeme NHR-25 u jedinců se Sys fenotypem, dochází k normálnímu vývoji gonády. Tento výsledek naznačuje, že pro správný vývoj gonády u *C. elegans* je důležitá rovnováha mezi aktivitou NHR-25 a signalizací Wnt.

V další části práce jsme se zaměřili na studium vzájemného vztahu mezi NHR-25 a signalizací Wnt. Data získaná prostřednictvím kvasinkového dvouhybridního systému naznačovala možnost interakce mezi NHR-25 a některými komponentami signalizace Wnt. Později jsme potvrdili, že NHR-25 je schopen přímo interagovat s oběma β-kateniny, které jsou důležité při vývoji gonády, tj. s WRM-1 a SYS-1. Schopnost vzájemné interakce mezi těmito proteiny je základem pro modulaci transkripční aktivity proteinu NHR-25 a rovněž tak heterokomplexu POP-1/SYS-1. Pomocí luciferázových reportérů jsme ukázali, že se jednotlivé β-kateniny liší ve svém vlivu na transkripci iniciovanou z NHR-25- senzitivních promotorů. Zatímco WRM-1 tuto transkripci inhibuje, tak SYS-1 působí jako aktivátor. Na druhé straně ovšem také protein NHR-25 významně působí na transkripci cílových promotorů signalizace Wnt. NHR-25 reprimuje transkripci mediovanou komplexy POP-1/SYS-1 (tj. komplexy Tcf/β-katenin).

Vliv jaderného receptoru NHR-25 na transkripci aktivovanou signalizací Wnt a naopak působení signalizace Wnt na transkripci mediovanou NHR-25 je pravděpodobně klíčovým

mechanismem při regulaci vývoje gonády u *C. elegans*. Porušení vzájemné rovnováhy mezi oběma procesy vede k vážným defektům, jak vyplývá z experimentů s nefyziologickou inaktivací NHR-25 a komponent signalizace Wnt. Modelově si můžeme představit dvě vzájemně kompatibilní situace (viz obr.6 v publikaci), jak by mohla interakce mezi NHR-25 a signalizací Wnt určovat vývoj prekurzorových buněk gonády.

První situace vychází z předpokladu, že NHR-25 působí jako modulátor signalizace Wnt a sám neaktivuje transkripci. V tomto případě v distálních buňkách udržuje WRM-1 spolu s kinázou LIT-1 nízkou jadernou koncentraci transkripčního faktoru POP-1, který tvoří heterokomplexy s limitním množstvím proteinu SYS-1, a tak dochází k transkripci distálně specifických genů. V distálních buňkách zároveň WRM-1 brání proteinu NHR-25 v represi transkripce POP1/SYS1-specifických genů. Naproti tomu v proximálních buněčných prekurzorech, NHR-25 blokuje transkripci distálně specifických genů, neboť na sebe váže protein SYS-1 a tím ještě zvyšuje množství volného transkripčního faktoru POP-1 v jádře. Volný POP-1 pak není schopen iniciovat transkripci. Je také možné, že NHR-25 inhibuje transkripci POP1/SYS1-specifických genů v proximální buňce svojí přímou interakcí s transkripčním komplexem.

Druhý možný způsob regulace vývoje gonády u *C. elegans* předpokládá existenci proximálně specifických genů, které ovšem doposud nebyly popsány. V tomto případě by v proximální buňce NHR-25 v komplexu spolu se SYS-1 inicioval transkripci proximálně specifických genů, zatímco volný POP-1 by reprimoval expresi genů distálních. Ovšem v distální buňce by signalizace Wnt prostřednictvím proteinu WRM-1 bránila transkripční aktivitě jaderného receptoru NHR-25, a tak by se veškerý SYS-1 v distální buňce vázal na POP-1 a heterokomplexy POP-1/SYS-1 by pak mohly iniciovat transkripci distálně specifických genů.

Předkládaná práce ukázala význam interakce mezi jaderným receptorem NHR-25 a různými β-kateniny a význam této interakce pro správný vývoj gonády u *C. elegans*. Ovšem tato interakce se zdá být evolučně poměrně konzervovanou. Synergická aktivita mezi β-kateninem a savčími orthology proteinu NHR-25 byla dokumentována několika pracemi. U savců existují orthology dva, a to protein LRH-1 (Liver Receptor Homolog 1) a SF-1 (Steroidogenic Factor 1). V případě LRH-1 vede jeho asociace s β-kateninem k zesílení transkripce specifických genů, a navíc LRH-1 je schopen svojí vazbou na β-katenin zesílit transkripci cílových genů signalizace Wnt. Oba tyto procesy vedou k aktivaci transkripce G_1 cyklinů a přispívají tím k indukci buněčné proliferace [225].

Vzájemná kooperace mezi jaderným receptorem SF-1 a β-kateninem (a tím i signalizací Wnt) je nezbytná při vývoji samčích pohlavních orgánů [226-228]. Rovněž jiné typy jaderných receptorů mohou interferovat se signální dráhou Wnt. Tak například androgenní receptor (AR) za určitých okolností kompetuje s transkripčním faktorem TCF-4 o stabilizovaný nukleární β-katenin, a tak blokuje signalizaci Wnt. Navíc komplexy AR/β-katenin jsou významnými aktivátory transkripce. Přestože se interakce mezi AR a komponentami signální dráhy Wnt může odehrávat ještě na několika dalších úrovních, je pravděpodobné, že má nezanedbatelný význam při vzniku některých prostatických nádorů [229-231].

Vyvážená interakce mezi signální drahou Wnt a aktivitou jaderných receptorů je tedy molekulárním mechanismem, který hraje významnou roli při regulaci řady vývojových procesů.

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Crosstalk between a Nuclear Receptor and β-Catenin Signaling Decides Cell Fates in the *C. elegans* Somatic Gonad

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Summary

β-Catenin signaling determines the proximal-distal axis of the *C. elegans* gonad by promoting distal fate in asymmetrically dividing somatic gonad precursor cells (SGPs). Impaired function of the Wnt effector POP-1/TCF, its coactivator SYS-1/β-catenin, and of upstream components including β-catenin WRM-1 causes all SGP daughters to adopt the proximal fate. Consequently, no distal tip cells (DTCs) that would lead differentiation of gonad arms form in the affected hermaphrodites. Here, we show that deficiency of the nuclear receptor NHR-25 has the opposite effect: extra DTCs develop instead of proximal cells. NHR-25 knockdown restores DTC formation and fertility in *pop-1* and *sys-1* mutants, suggesting that a balance between NHR-25 and β-catenin pathway activities is required to establish both proximal and distal fates. This balance relies on direct crossregulation between NHR-25 and the distinct β-catenin proteins WRM-1 and SYS-1. The nuclear receptor-β-catenin interaction may be an ancient mechanism of cell-fate decision.

Introduction

Diverse cell types can arise either from initially identical daughter cells that receive different instructions or by intrinsically asymmetric cell divisions whereby the two daughters inherit different molecular determinants and with them unequal developmental potentials (Kemphues, 2000; Betschinger and Knoblich, 2004; Roegiers and Jan, 2004). Conserved transduction pathways, such as Wnt signaling (Logan and Nusse, 2004), are critical for cell-fate decisions. In canonical cascades, a Wnt glycoprotein signals its receptor to stabilize β-catenin, which in turn cooperates with TCF/LEF transcription factors to activate a specific set of genes; in the absence of the Wnt signal, TCF/LEF proteins repress these genes. In *C. elegans*, polarity of asymmetric cell divisions is dictated by noncanonical Wnt pathways involving distinct β-catenins WRM-1 and SYS-1 and a MAPK-related kinase, LIT-1 (Thorpe et al., 1997; Rocheleau et al., 1997, 1999; Meneghini et al., 1999; Ishitani et al., 1999; Korswagen et al., 2000; Korswagen, 2002; Siegfried and Kimble, 2002; Herman and Wu, 2004; Kidd et al., 2005). These pathways converge at the TCF/LEF homolog POP-1, whose asymmetric distribution is key to the polarity of cell fates (Lin et al., 1995, 1998; Meneghini et al., 1999; Herman, 2001; Maduro et al., 2002; Lo et al., 2004; Siegfried et al., 2004; Kidd et al., 2005; Shetty et al., 2005).

The gonad of *C. elegans* is a remarkable example of how a single asymmetric cell division establishes the axis of the entire organ. The gonad develops from two somatic gonad precursors (SGPs) Z1 and Z4 that flank germline founder cells Z2 and Z3 (Kimble and Hirsh, 1979) (Figure 1A). Distal daughters of Z1 and Z4 cells by their next division produce a unique distal tip cell (DTC), one per each lineage. In hermaphrodites, the two DTCs reside at the opposite poles of the gonad and guide elongation of its arms and proliferation of the germline. Thus, each gonadal arm is organized along a proximal-distal axis. The proximal SGP daughters divide twice to produce cells of which one is selected as the anchor cell (AC) (Figure 1A) that will induce the vulva (Kimble, 1981).

The current model for axis formation in the somatic gonad (Kidd et al., 2005) relies on asymmetric distribution of POP-1 between the immediate Z1/Z4 daughter cells (Siegfried et al., 2004). Based on evidence from the early embryo, this asymmetry can be generated by LIT-1- and WRM-1-dependent nuclear export of POP-1 (Rocheleau et al., 1999; Maduro et al., 2002; Lo et al., 2004) in the distal daughters, where the remaining nuclear POP-1 combines with limiting amounts of β-catenin SYS-1 to activate distal fate genes. One such POP-1 target required for DTC formation is a Hox gene, *ceh-22/tinman* (Lam et al., 2006), originally uncovered as *sys-3* (Siegfried et al., 2004). In the proximal daughters, SYS-1-free nuclear POP-1 is thought to be a repressor (Kidd et al., 2005). Impaired function of *lit-1*, *wrm-1*, *pop-1*, *sys-1*, or *ceh-22* leads to a Sys (symmetrical sisters) phenotype, when all SGP daughters adopt the proximal fate, thus increasing the number of AC precursors at the expense of DTCs (Miskowski et al., 2001; Siegfried and Kimble, 2002; Siegfried et al., 2004). Conversely, misexpression of SYS-1 or CEH-22 transforms proximal cells into extra DTCs (Kidd et al., 2005; Lam et al., 2006). As no factor that would be required for the proximal fate commitment has yet been reported, the proximal fate appears to be a signaling-independent "default state."

Here, we show that the establishment of both the distal and the proximal cell fates requires a proper balance between POP-1 activity and the action of the nuclear receptor NHR-25. Depletion of NHR-25 causes extra DTCs to form at the expense of the proximal AC precursors and reverts the Sys phenotype of the β-catenin/MAPK pathway mutants. NHR-25 antagonizes POP-1- and SYS-1-dependent transcription, while WRM-1 inhibits and SYS-1 stimulates activation by NHR-25. Since

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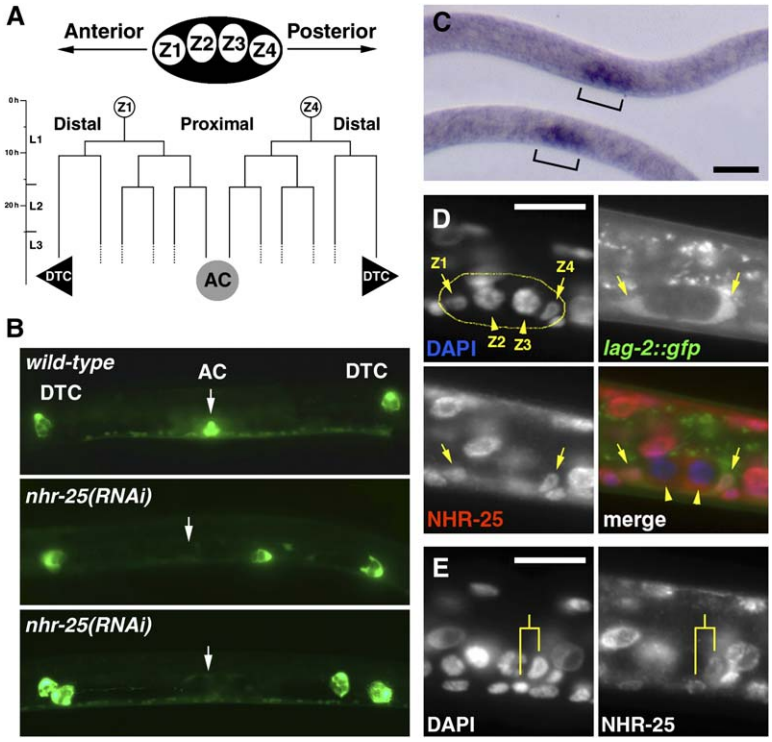

Figure 1. Excessive DTCs Form Instead of the Proximal Anchor Cell in *nhr-25(RNAi)* Hermaphrodites

(A) The asymmetric division of the somatic gonad precursors Z1 and Z4 occurs during the L1 stage at around 8 hr posthatching (Kimble and Hirsh, 1979). The following divisions produce two distal tip cells (DTC) and proximal cells, one of which is selected as the anchor cell (AC).

(B) Two DTCs and one AC in a normal worm (top) are visualized by *lag-2::gfp* and *cdh-3::gfp* markers, respectively. *nhr-25* RNAi applied by feeding since the time of hatching causes one (middle) or two (bottom) extra DTCs to form at the expense of the AC (arrows indicate the normal AC position).

(C) In situ hybridization reveals that *nhr-25* mRNA is expressed in the early gonad primordium (brackets). Bar = 20 μm.

(D) The endogenous NHR-25 protein is detected in the nuclei of Z1 and Z4 (arrows) but not in the Z2/Z3 germline precursors (arrowheads). The yellow line shows the approximate shape of the gonad primordium, and *lag-2::gfp* expression marks Z1 and Z4 cells.

(E) NHR-25 is also present in the immediate Z4 (yellow lines) and Z1 (out of focus) daughters. Bars in (D) and (E) = 10 μm. In all panels, anterior is to the left.

NHR-25 belongs to an ancient nuclear receptor family whose members functionally interact with β-catenin in mammals, the crosstalk between NHR-25 and β-catenin signaling, uncovered in the *C. elegans* gonad, may present a broadly conserved principle of cell-fate decision.

Results

Impaired *nhr-25* Function Causes an "All-Distal" Sys Phenotype

Unlike normal hermaphrodites with two DTCs, up to 55% of worms that were subjected to *nhr-25* RNAi silencing from the time of hatching developed three or four DTCs (Figure 1, Table 1, and Table S1). The ectopic DTCs could not be distinguished from normal DTCs by morphology or expression of a transgenic GFP marker derived from the *Delta*-like gene *lag-2* (Blelloch et al., 1999) (Figure 1B). Consistently, these cells were functional in promoting germline differentiation and in leading the migration of gonadal arms; in case of four DTCs, two arms extended in the anterior and two in the posterior direction. The total number of DTCs never exceeding four and the simultaneous loss of the AC (Figure 1B and Table S1) argued that *nhr-25* RNAi disrupted the asymmetry of the fate-determining Z1/Z4 division that occurs at about 8 hr posthatching (Kimble and Hirsh, 1979) (Figure 1A) and not by causing proliferation of already distally specified cells. To confirm this idea, we applied *nhr-25* RNAi at increasing intervals after hatching. The latest time at which the DTC number could be altered (three DTCs in 3% of animals, n = 196) was 4 hr posthatching; starting from 7 hr, it was too late for *nhr-25* RNAi to affect the critical Z1/Z4 division (Table S1).

Table 1. *nhr-25* Genetically Interacts with *wrm-1* and *lit-1* during Gonad Development

RNAi Targets[a]		DTC Number (% Animals)		
Injection	Feeding	Missing (0–1)	Extra (3–4)	n
None	empty vector	1	0	516
	nhr-25	6[b]	55	228
wrm-1	empty vector	91	0	214
	nhr-25	27	14	193
lit-1	empty vector	23	0	66
	nhr-25	3	14	347

[a] Homozygous *rde-1* hermaphrodites were injected with either *wrm-1* or *lit-1* dsRNA and mated to *rde-1*$^{+/+}$ males carrying the *lag-2::gfp* DTC marker on plates seeded with either control bacteria with the empty pPD129.36 vector or bacteria expressing *nhr-25* dsRNA.
[b] Maternal contribution of *nhr-25* dsRNA prevents formation of the gonad primordium.

To verify the specificity of the *nhr-25* RNAi effect, we repeated feeding with dsRNA spanning two nonoverlapping regions of the *nhr-25* mRNA. Regardless of whether the region included the conserved DNA binding domain, the same gonad phenotype was observed (data not shown). Moreover, we used a related nuclear receptor gene, *nhr-23*, for control. While *nhr-23* RNAi caused expected Dumpy (Dpy) defects (Kostrouchova et al., 1998), it could not elicit the extra DTC phenotype at any time.

Because silencing of *nhr-25* causes extra DTCs to form at the expense of proximal Z1/Z4 descendants, it disrupts the gonad asymmetry in a sense opposite to all thus far reported Sys defects. These results therefore suggest that the normal role of NHR-25 is to promote the proximal fate or to antagonize the distal fate. Consistent with such a role, *nhr-25* mRNA is present in the SGPs (Figure 1C). The NHR-25 protein can be detected in the nuclei of Z1 and Z4 (Figure 1D) as well as in the nuclei of their daughter cells (Figure 1E), but not in the Z2 and Z3 precursors of the germline.

nhr-25 Antagonizes *pop-1* and Other β-Catenin/MAPK Pathway Genes

While *nhr-25* is required for the specification of the proximal fate, *pop-1*, *sys-1*, *wrm-1*, and *lit-1* all promote the distal fate. To test for genetic interaction between *nhr-25* and the β-catenin/MAPK pathway, we primarily chose a *pop-1* mutation *(q645)* and a *sys-1* mutation *(q544)*, which both are fully penetrant for the gonadal Sys defect (Miskowski et al., 2001; Siegfried and Kimble, 2002). Because all SGP daughters become proximally specified and none yields a DTC in either *pop-1(q645)* or *sys-1(q544)* homozygous hermaphrodites, all adults lack both gonadal arms and are sterile. We found that *nhr-25* silencing applied from the time of hatching (but not after the critical Z1/Z4 division; Table S2) allowed 61% (n = 44) of *pop-1(q645)* hermaphrodites to develop one or both gonadal arms, i.e., clear evidence that DTCs also were formed (Figure 2). In addition, fertility was restored in 23 out of 141 examined *pop-1(q645); nhr-25(RNAi)* adults, indicating that the germline differentiated properly. The same suppression of the Sys phenotype was also observed in *sys-1(q544)* mutants subjected to *nhr-25* RNAi (Figure 2 and Table S2). This effect was specific to *nhr-25*, since the Sys phenotype of neither mutant could be reversed by RNAi targeting of the related nuclear receptor NHR-23 (data not shown).

The observed suppression was striking since the *pop-1(q645)* mutation was 100% penetrant for the Sys defect in animals with undisturbed *nhr-25* function. It implied that the MAPK/β-catenin pathway, which is essential for DTC formation, can signal even in the *pop-1(q645)* background. This finding and also the fact that the *pop-1(q645)* allele caused a single conservative substitution of aspartic acid 9 to glutamic acid (D9E) (Siegfried and Kimble, 2002) prompted us to examine whether the POP-1(q645) mutant protein might be partly functional. We therefore tested POP-1(q645) along with a nonconservative mutant version, POP-1(D9A), for the ability to activate a TCF-dependent pTOPFLASH reporter in SYS-1/β-catenin-dependent manner. Whereas the POP-1(D9A) protein did not activate at all, the ability of POP-1(q645) to induce pTOPFLASH expression in the presence of SYS-1 was only lowered as compared to wild-type POP-1 (Figure 3).

Simultaneous RNAi silencing of *pop-1* and *nhr-25* reduced the effect of *pop-1* RNAi on DTC loss as well as the effect of *nhr-25* RNAi on DTC surplus, respectively (Table S3). In comparison to *pop-1* RNAi, *sys-1* knockdown was more effective in suppressing the extra DTC phenotype caused by *nhr-25* RNAi. Conversely, silencing of *nhr-25* only mildly suppressed the effect of *sys-1* RNAi on DTC loss (Table S3). Since *sys-1* is dose-sensitive and haploinsufficient (Kidd et al., 2005), these differences may reflect a relatively higher sensitivity of *sys-1* to RNAi.

The above results further confirmed that NHR-25 antagonized POP-1 and SYS-1. A similar relationship was also found between *nhr-25* and genes encoding the kinase LIT-1 and the β-catenin WRM-1 (Table 1). *nhr-25* RNAi reduced to about one-third the effect of *wrm-1* RNAi and abolished the effect of *lit-1* RNAi on DTC loss. Conversely, silencing of either *wrm-1* or *lit-1* reduced frequency of the extra DTC phenotype caused by *nhr-25* RNAi from 55% to 14% (Table 1). Together,

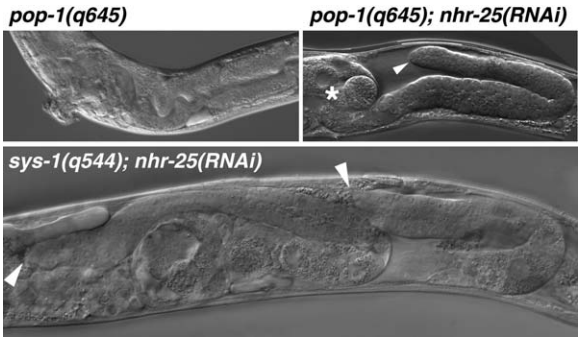

Figure 2. *nhr-25* Counteracts *pop-1* and *sys-1* during Gonad Development

The absence of DTCs and hence of the gonad arms caused in hermaphrodites by *pop-1(q645)* (top left) or by *sys-1(q544)* (not shown) (Miskowski et al., 2001) mutations can be reverted by *nhr-25* RNAi. Three DTCs may develop and consequently two gonadal arms migrate in the same direction in some *sys-1(q544); nhr-25(RNAi)* (bottom) or *pop-1(q645); nhr-25(RNAi)* (not shown) hermaphrodites. Arrowheads point to DTCs; asterisk indicates an embryo.

these results suggested that NHR-25 opposed β-catenin/MAPK signaling during cell fate decision in the somatic gonad precursors. Since *nhr-25* RNAi did not appear to alter the distribution of a transgenic GFP::POP-1 fusion protein (Siegfried et al., 2004) (Figure S1), NHR-25 likely acted in parallel to β-catenin/MAPK signaling and POP-1.

NHR-25 Binds β-Catenins WRM-1 and SYS-1

We next asked whether NHR-25 interacts with the β-catenin/MAPK pathway by making direct contact with some of its components. Yeast two-hybrid assay

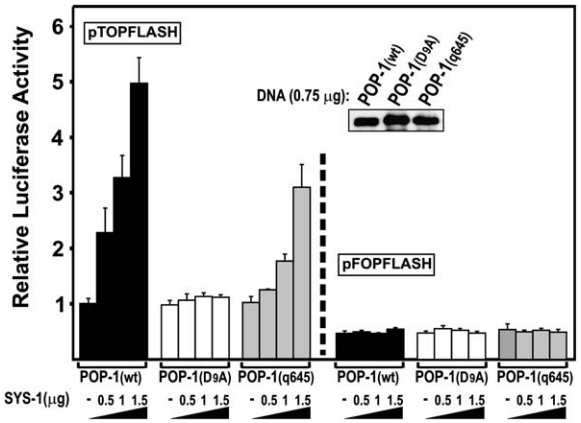

Figure 3. POP-1(q645) Mutant Protein Retains Partial Function

When transfected to HEK239 cells, the POP-1(q645) mutant bearing a D9E substitution could still activate the pTOPFLASH reporter in a SYS-1/β-catenin-dependent manner (gray columns), albeit at a reduced rate compared to wild-type POP-1 (black columns). Mutation of D9 to alanine (D9A) abolished the activation completely (open columns). Inset shows that protein expression levels did not differ among the three POP-1 variants. pFOPFLASH was used for negative control. Luciferase activities normalized for the *Renilla* internal control are expressed relative to the activity of wild-type POP-1 in the absence of SYS-1, arbitrarily set to 1. Values are average plus standard deviation of three independent experiments, each of which was done in triplicate; the amounts of transfected plasmid DNAs are given for the entire triplicate experiment.

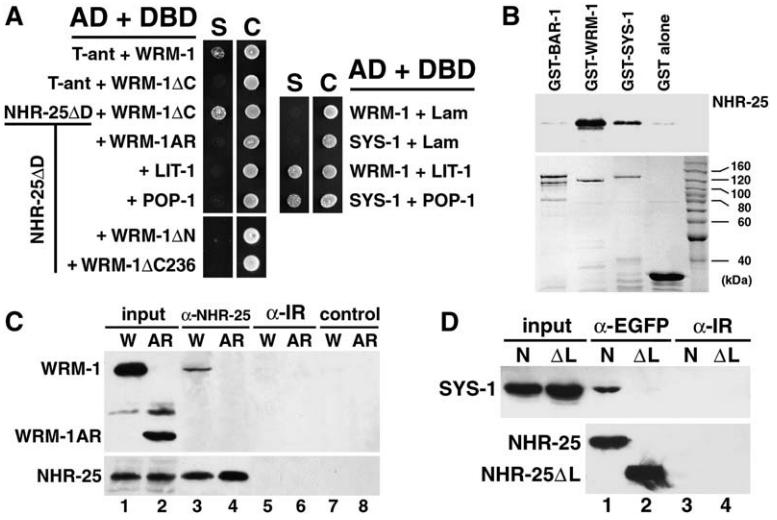

Figure 4. NHR-25 Interacts with β-Catenins WRM-1 and SYS-1

(A) In yeast two-hybrid assay, deletion of 23 C-terminal amino acids prevented reporter activation by WRM-1 in the presence of a control (T-antigen), but not its interaction with NHR-25ΔD (lacking the DNA binding domain). Removal of either the 216 N-terminal amino acids (WRM-1ΔN), of the C terminus (WRM-1ΔC236) or both (WRM-1AR) abolished the interaction. LIT-1 and POP-1 bound their partners, WRM-1 and SYS-1, respectively, but neither bound NHR-25ΔD. In all assays, fusions with the Gal4 activation domain (AD) are on the left. S, selective conditions; C, control plate.

(B) In vitro-translated and ^{35}S-labeled NHR-25 was pulled down in complexes with bacterially produced GST-WRM-1 and GST-SYS-1 fusion proteins, but not with another β-catenin BAR-1. Bottom gel shows Coomassie blue staining of the GST fusion proteins.

(C) NHR-25 immunoprecipitated with full-length WRM-1 (W, lane 3) but not with its truncated version WRM-1(AR) (AR, lane 4). Lysates from transfected HEK239 cells were precipitated with anti-NHR-25 or an unrelated (α-IR) antibody. Control cells received no NHR-25 (lanes 7 and 8). Epitope-tagged WRM-1 proteins were detected with α-Myc antibody.

(D) SYS-1 (detected with α-Myc) immunoprecipitated with NHR-25 (N, lane 1) but not with NHR-25ΔL lacking the ligand binding domain (ΔL, lane 2). Because the NHR-25 antibody is directed against the deleted ligand binding domain (Silhankova et al., 2005), NHR-25 was tagged with EGFP to allow immunoprecipitation and detection with an anti-EGFP antibody. No complex was recovered from the transfected cells with the control α-IR antibody.

revealed binding of NHR-25 to β-catenin/WRM-1 but not to POP-1 or LIT-1 proteins (Figure 4A); SYS-1 could not be tested because when fused to the Gal4 DNA binding domain, it strongly activated gene expression in the yeast. WRM-1 was only a moderate transcriptional activator, and by deleting 23 amino acids from its carboxyl end, we obtained WRM-1ΔC that did not activate alone but still interacted with NHR-25 (Figure 4A). Removal of either the N-terminal 216 amino acids (WRM-1ΔN) or the C-terminal 236 amino acids from WRM-1 abolished the interaction. WRM-1 lacking both ends and containing the armadillo repeats (WRM-1AR) was also incapable of binding NHR-25 (Figure 4A). GST pull-down with in vitro-translated NHR-25 and bacterially produced β-catenin proteins was performed to confirm that the binding was direct. In this assay, NHR-25 bound WRM-1 and SYS-1 but not another *C. elegans* β-catenin, BAR-1 (Figure 4B). Finally, interaction of NHR-25 with WRM-1 and SYS-1 was verified by coimmunoprecipitation in transfected cell culture. Consistent with the yeast two-hybrid data, WRM-1 but not WRM-1AR could be immunoprecipitated with NHR-25 by using a NHR-25-specific antibody (Figure 4C, lanes 3 and 4). SYS-1 coimmunoprecipitated with NHR-25, but not with its C-terminally truncated version lacking the ligand binding domain (NHR-25ΔL) (Figure 4D, lanes 1 and 2). As the NHR-25-specific antibody reacted poorly with the truncated NHR-25 protein, EGFP-tagged proteins and an anti-EGFP antibody were used in these experiments. Nevertheless, coimmunoprecipitation with SYS-1 was specific to full-length NHR-25 because in a parallel assay, neither EGFP-NHR-25ΔL nor a control, EGFP protein, bound to SYS-1 (Figure 4D and data not shown).

Crosstalk between NHR-25 and β-Catenins WRM-1 and SYS-1 at the Level of Transcriptional Activation

The antagonism between NHR-25 and β-catenin/MAPK signaling that was uncovered by genetic experiments suggested that WRM-1 might be a negative regulator of NHR-25. We tested the relationship between NHR-25 and WRM-1 in cell transfection assays with NHR-25-dependent luciferase reporters, one containing a tandem of Ftz-F1/SF-1 consensus binding sites (designated 2×TCA) or their mutated version 2×TCT (Figure 5A), and the other based on five SF-1 binding sites from the mouse *CYP21* gene (Figure 5B). NHR-25 alone stimulated transcription of both reporters in a dose-dependent manner (not shown and Figure 5B). Activation by an intermediate dose of NHR-25 was progressively suppressed by increasing doses of WRM-1 (Figures 5A and 5B), while the truncated WRM-1AR version that failed to bind NHR-25 (Figures 4A and 4C) had no effect. In contrast, the other β-catenin SYS-1 stimulated NHR-25-dependent transactivation of both reporters (Figures 5A and 5B). Thus, the two functionally distinct β-catenins modulated NHR-25 activity in opposite ways.

In addition to being regulated by WRM-1 and SYS-1, NHR-25 itself might antagonize POP-1 by interfering with transcription activated by the POP-1/SYS-1 nuclear complex. We verified this possibility by using the pTOP-FLASH reporter. While full-length NHR-25 clearly inhibited pTOPFLASH activation by POP-1 and SYS-1, NHR-25 lacking its ligand binding domain (NHR-25ΔL) had no effect (Figure 5C). The inhibition likely occurred through NHR-25 binding to SYS-1, since full-length NHR-25 but not NHR-25ΔL coimmunoprecipitated with SYS-1 (Figure 4D, lanes 1 and 2).

Discussion

Role of NHR-25 in the Early Somatic Gonad

The effect of NHR-25 in the early gonad opposed that of MAPK/β-catenin signaling because only distal cells formed when NHR-25 function was reduced, and only proximal cells resulted from the compromised

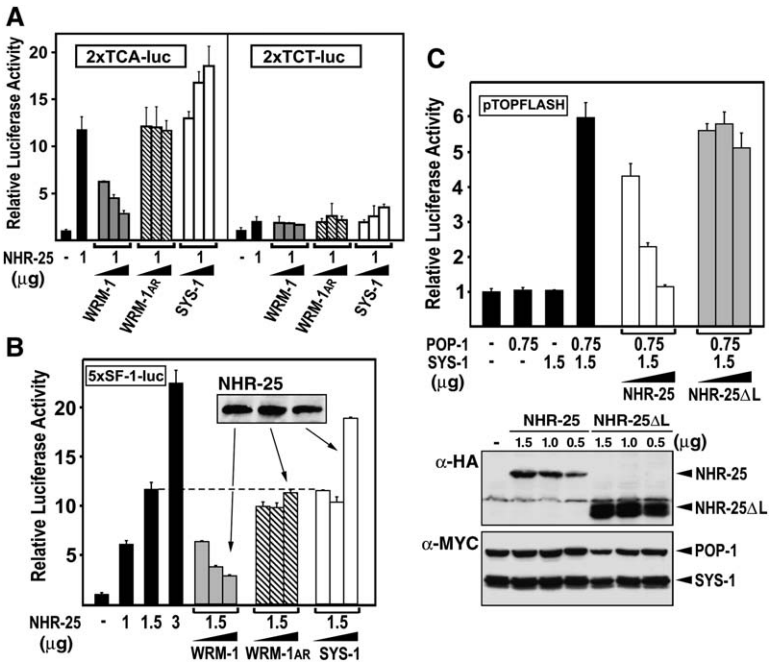

Figure 5. Crossregulation between β-Catenin, NHR-25, and POP-1

(A and B) WRM-1 inhibits and SYS-1 stimulates NHR-25-dependent transcription. HEK293 cells were cotransfected with an NHR-25 expression plasmid and either with luciferase reporters driven by two Ftz-F1/SF-1 consensus binding elements (2×TCA) and their mutant version (2×TCT) (A) or with the 5×SF-1-luc reporter (B). Activation by intermediate NHR-25 levels (broken line in B) was suppressed by increasing doses (0.5, 1.0, and 1.5 μg DNA) of WRM-1 (gray columns), but not by the truncated WRM-1AR (hatched columns). SYS-1 (open columns) enhanced NHR-25-dependent activation of both 2×TCA and 5×SF-1-luc reporters. Inset in (B) shows that NHR-25 expression did not change among experiments.

(C) NHR-25 interferes with POP-1/SYS-1 activity. pTOPFLASH activation by cotransfected POP-1 and SYS-1 was inhibited by increasing doses (0.5, 1.0, and 1.5 μg DNA) of NHR-25 (open columns) but not by NHR-25ΔL (gray columns). The inhibition was not due to altered POP-1 or SYS-1 expression (bottom). In all panels, luciferase activities corrected for the *Renilla* internal control are expressed relative to reporter activities in control cells, arbitrarily set to 1. Values are average plus standard deviation of three independent experiments, each of which was done in triplicate; the microgram amounts of transfected plasmid DNAs are given for the entire triplicate experiment.

MAPK/β-catenin pathway activity. Perturbing NHR-25 and this pathway simultaneously permitted both the distal and proximal fates to be established. Strikingly, depletion of NHR-25 restored gonad differentiation in *pop-1(q645)* mutants that otherwise never develop DTCs (Siegfried and Kimble, 2002). Although mutation of the invariably conserved aspartate to a glutamate residue disrupted POP-1 binding to SYS-1/β-catenin in yeast (Kidd et al., 2005), we suspected that the POP-1(q645) mutant protein was still partly active. That this was indeed true was not surprising given the conservative nature of the substitution and the fact that POP-1(q645) weakly bound β-catenin BAR-1 (Kidd et al., 2005). Interestingly, *ceh-22b::VENUS* expression was not completely abolished in *pop-1(q645)* worms (Lam et al., 2006), indicating that the mutant protein was also partly functional in vivo.

Since neither the mutationally induced POP-1 defect nor RNAi knockdown of *nhr-25* (whose deletion is embryonic lethal, Asahina et al., 2000) represent complete loss-of-function situations, epistasis between NHR-25 and POP-1 is not obvious. Like SYS-1, NHR-25 does not seem to be required for POP-1 asymmetry. In fact, NHR-25 role in POP-1 distribution between the SGP daughters may not be expected since neither equal POP-1 distribution (Siegfried et al., 2004) nor its reversed asymmetry (Chang et al., 2005) transforms proximal cells to distal. Defects in the MAPK/β-catenin pathway and NHR-25 complement each other: whereas *nhr-25* knockdown allows more cells to become DTCs in *pop-1*, *sys-1*, *lit-1*, or *wrm-1* RNAi backgrounds, silencing of any of these genes reduces the frequency of extra DTCs caused by shortage of NHR-25. In our view, these interactions reflect a mutual balance that must exist between competing NHR-25 and MAPK/β-catenin programs in order for both fates to be established.

To explain how this balance may be achieved, we propose two alternative but compatible models (Figure 6). Both models rely on unequal distribution of WRM-1 and LIT-1 between daughters of asymmetric cell divisions, which has been demonstrated in the early embryo (Lo et al., 2004; Nakamura et al., 2005) and in several cell types during postembryonic development (Takeshita

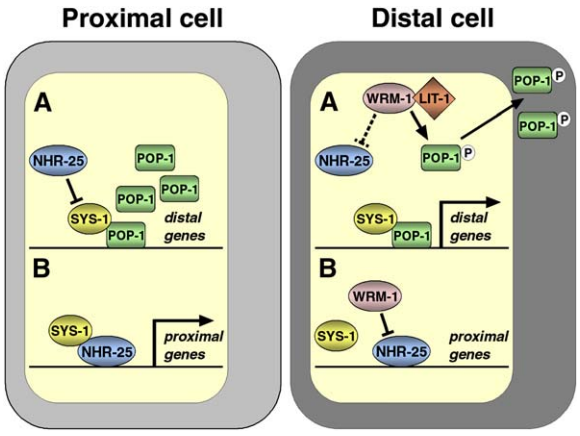

Figure 6. Model for NHR-25 Interaction with the β-Catenin/MAPK Pathway during Cell-Fate Specification of the Somatic Gonad Precursors

(A) NHR-25 promotes proximal fate as a negative modulator of SYS-1/POP-1 activity in the proximal Z1/Z4 daughter cell. In the distal daughter, WRM-1 and LIT-1 mediate nuclear export of excess POP-1, allowing the remaining POP-1 to combine with SYS-1 and to activate distal genes (based on the model in Kidd et al., 2005). NHR-25 may be inactivated by WRM-1 binding (dotted line).

(B) NHR-25 promotes proximal fate by regulating its own target gene(s). SYS-1 enhances NHR-25 activity. In the distal cell, WRM-1 prevents NHR-25-dependent activation of "proximal genes." Both the A and B models may apply together.

and Sawa, 2005). In all cases (including the distal daughters of Z1 and Z4, H. Sawa, personal communication), WRM-1 and LIT-1 accumulate in the nuclei of those cells that display less nuclear POP-1, presumably due to LIT-1- and WRM-1-mediated nuclear export of POP-1 (Rocheleau et al., 1999; Lo et al., 2004).

In model A, NHR-25 ensures the correct fate of the proximal SGP daughter by preventing POP-1/SYS-1-dependent activation of distal genes (Figure 6). In the distal cell, WRM-1 plays two roles. First, as proposed by Kidd and colleagues, WRM-1 and LIT-1 mediate nuclear export of excess POP-1, allowing the remaining POP-1/SYS-1 complex to trigger distal genes (Kidd et al., 2005). Second, WRM-1 may prevent NHR-25 from binding SYS-1 and thus from inhibiting POP-1 activation. Whether WRM-1 and SYS-1 compete for NHR-25 binding remains to be tested, but this idea is suggested by apparently stronger NHR-25 interaction with WRM-1 as compared to SYS-1 in vitro (Figure 4B). If WRM-1 prevails in the distal cell nucleus, NHR-25 would be more likely combined with it rather than with SYS-1. Model B predicts that the absence of nuclear WRM-1 in the proximal cell allows NHR-25 to turn on its own proximal target gene(s). In this case, SYS-1 can enhance NHR-25 activity. In the distal cell, WRM-1 would prevent NHR-25-dependent activation of these proximal genes. Since we have not yet identified any direct NHR-25 target, there is no definite proof for model B.

Future studies on NHR-25 may help us resolve some open questions about gonad development. According to the recent model (Kidd et al., 2005), high nuclear POP-1 is a repressor of distal genes in the proximal SGP daughter. However, POP-1-dependent repression could not be demonstrated for the freshly characterized distal gene *ceh-22b* (Lam et al., 2006). Whether NHR-25 represses *ceh-22b* or whether it acts upon POP-1-independent targets needs to be tested. Another question concerns a missing signal that would polarize the early somatic gonad. Although upstream Wnt signaling components such as LIN-17/Frizzled (Sternberg and Horvitz, 1988; Siegfried and Kimble, 2002) and DSH-2/Dishevelled (Chang et al., 2005) contribute to SGP asymmetry, none of the five known *C. elegans* Wnts appears to be required for it (Siegfried and Kimble, 2002). Recently, mammalian NHR-25 homologs have been shown to bind phospholipid ligands (Krylova et al., 2005; Li et al., 2005). If NHR-25 requires an activating ligand, then this molecule, rather than a Wnt, might be the missing polarizing cue for the somatic gonad precursors.

NHR-25 Interaction with β-Catenin Signaling Is Evolutionarily Conserved

The present study introduces NHR-25 as a new player and, to our knowledge, as the first *C. elegans* nuclear receptor to be involved in β-catenin signaling. Homologs of NHR-25, the steroidogenic factor 1 (SF-1) and the liver receptor homolog 1 (LRH-1), have been shown to interact with β-catenin in mammalian models. Thus, a functional relationship between β-catenin and the NR5 family of nuclear receptors is common to worms and mammals.

Synergy between SF-1 and β-catenin is important for male sexual differentiation, e.g., for TCF4-dependent expression of a Müllerian inhibiting substance receptor (Hossain and Saunders, 2003). Conversely, the β-catenin-SF-1 complex stimulates SF-1-dependent activation of the *Dax-1* (Mizusaki et al., 2003) and *α-inhibin* (Gummow et al., 2003) genes. Interestingly, the Wnt4 signal opposes male sexual development by repressing the cooperation between β-catenin and SF-1 (Jordan et al., 2003). A two-way synergy between β-catenin and LRH-1 has been proposed to stimulate cell proliferation in mice: whereas β-catenin enhances expression of an LRH-1 target gene, *cyclin E1*, LRH-1 serves as a coactivator to the TCF4-β-catenin complex on the *cyclin D1* promoter (Botrugno et al., 2004). This LRH-1-TCF4 crossregulation perhaps best parallels a scenario in which NHR-25 and POP-1 act on their own target genes (Figure 6).

In the light of the above examples of mutual synergy, it was somewhat surprising that NHR-25 inhibited POP-1- and SYS-1-dependent gene expression, whereas WRM-1 inhibited the transcriptional activity of NHR-25. One reason may be that in contrast to mammals, the noncanonical *C. elegans* pathway employs structurally unusual and functionally distinct β-catenin molecules (Korswagen et al., 2000; Natarajan et al., 2001; Kidd et al., 2005). Unlike WRM-1, SYS-1 (which serves as coactivator to POP-1) stimulated NHR-25-dependent reporter expression, a situation similar to the synergy between β-catenin and SF-1 (Gummow et al., 2003; Hossain and Saunders, 2003; Jordan et al., 2003; Mizusaki et al., 2003) or LRH-1 (Botrugno et al., 2004). Additionally, Gummow and colleagues showed that increased levels of TCF4 disrupted the synergy between SF-1 and β-catenin on the SF-1-responsive *α-inhibin* promoter (Gummow et al., 2003). Therefore, both positive and negative regulation can occur between nuclear receptors NHR-25 or SF-1 and β-catenin signaling.

Accumulating literature shows that modulation between Wnt/β-catenin signaling and numerous types of nuclear receptors proceeds in both directions: nuclear receptors block or stimulate Wnt signaling, while Wnt pathways positively or negatively affect the transcriptional activity of nuclear receptors (reviewed in Mulholland et al., 2005). This regulatory interplay emerges as an important means of controlling cell differentiation. Our study shows that the β-catenin-nuclear receptor crosstalk has been conserved in distant animal phyla and that the *C. elegans* system allows us to link it with developmental decisions of specific cells in vivo.

Experimental Procedures

Worms and RNA Interference

C. elegans was reared at 20ºC according to standard protocols (Brenner, 1974). The following strains were used. GS2806 *dpy-20(e1362)*; *arIs51[dpy-20(+)*; *cdh-3::gfp]* (Pettitt et al., 1996) strain was a gift from I. Greenwald (Columbia University, New York, NY). JK3785 *sys-1(q544)/hT2(qIs48); qIs90* (Lam et al., 2006) was kindly provided by J. Kimble (University of Wisconsin, Madison, WI). JK2868 *qIs56[lag-2::gfp]* (Blelloch et al., 1999), JK2944 *pop-1(q645)* (Siegfried and Kimble, 2002), JK3437 *him-5(e1490); qIs74* (Siegfried et al., 2004), and WM27 *rde-1(ne219)* (Tabara et al., 1999) were obtained from the *Caenorhabditis* Genetics Center, supported by the National Institutes of Health. GS2806 and JK2868 were crossed to generate a strain carrying both *cdh-3::gfp* and *lag-2::gfp*. The N2 variety Bristol was used as the wild-type.

RNA interference (RNAi) by feeding was essentially performed as described (Timmons et al., 2001). pPD129.36 vector (a gift from A. Fire, Carnegie Institution of Washington, Baltimore, MD) was

used for dsRNA expression in *Escherichia coli* HT115 (a gift from D. Court, National Cancer Institute, Frederick, MD). Bacteria carrying a pPD129.36 construct were grown to OD_{600} = 0.4 in LB medium containing 50 μg/ml carbenicillin and 12.5 μg/ml tetracycline, and dsRNA was induced for 4 hr with 0.4 mM isopropyl-β-D-thiogalactoside (IPTG). Bacteria were concentrated by centrifugation and seeded on nematode growth medium (NGM) agarose plates containing both antibiotics and 0.4 mM IPTG. Bacteria carrying pPD129.36 without an insert were used for control.

To synchronize the worms, eggs from hermaphrodites were harvested by using the hypochlorite method and were placed on non-seeded NGM plates. Larvae that hatched within the first 2 hr were discarded. Fresh L1 larvae that hatched within 30 min were then collected, transferred to seeded plates, and kept at 20ºC. For RNAi applied at later times than immediately from hatching, the fresh L1 larvae were first fed (for 4, 7, 10, or 20 hr) on plates seeded with IPTG-induced control HT115 bacteria carrying the empty pPD129.36 vector and then transferred to dsRNA-expressing bacteria.

For double-target RNAi, cultures of *E. coli* expressing dsRNAs were pooled at equal ratio of cells, concentrated by centrifugation and seeded. Alternatively, zygotic RNAi was performed by injecting *rde-1(ne219)* (Tabara et al., 1999) hermaphrodites with in vitro-synthesized *wrm-1* or *lit-1* dsRNA. Injected worms were then mated with wild-type males carrying the *lag-2::gfp* transgene on plates seeded either with IPTG-induced bacteria containing the empty pPD129.36 vector or on bacteria that expressed *nhr-25* dsRNA.

Plasmid Constructs

The following yk clones were used to prepare RNAi constructs in pPD129.36: yk342d8 (*nhr-25*), yk1529d11 (*lit-1*), yk1201e5 (*wrm-1*). For *pop-1* and *sys-1* RNAi constructs, cDNAs were amplified from N2 worms by using RT-PCR.

cDNAs containing the complete open-reading frames of each gene were obtained from N2 worms by using RT-PCR and were sequenced in their entirety before cloning into expression vectors. Deletion mutants of each protein were generated either by subcloning restriction fragments of the cDNAs or by PCR with internally positioned primers; in the latter case, the resulting DNA was resequenced. Plasmids for yeast two-hybrid assays were prepared in the pGADT7 (Gal4AD fusion) and pGBKT7 (Gal4DBD fusion) vectors (Clontech). The Gal4AD fusion vectors included: pGADT7(T-ant), control construct with SV40 large T-antigen; pGADT7(NHR-25ΔD), NHR-25(117–572), deletion of the DNA binding domain; pGADT7(WRM-1), full-length WRM-1; and pGADT7(SYS-1), full-length SYS-1. The Gal4DBD fusion vectors included: pGBKT7(Lam), control construct with human lamin C; pGBKT7(WRM-1), full-length WRM-1; pGBKT7(WRM-1ΔC), WRM-1(1–773), deletion of the 23 C-terminal amino acids; pGBKT7(WRM-1ΔN), WRM-1(217–796), deletion of the 216 N-terminal amino acids; pGBKT7(WRM-1ΔC236), WRM-1(1–560), deletion of the 236 C-terminal amino acids; pGBKT7(WRM-1AR), WRM-1(135–560), deletion of the 134 N-terminal and of the 236 C-terminal amino acids; pGBKT7(POP-1), full-length POP-1; and pGBKT7(LIT-1), full-length LIT-1.

For transfections of mammalian cells, the following constructs encoding Myc epitope-tagged proteins were prepared in the pK-Myc-C3 vector (Valenta et al., 2003): pK-Myc-C3(SYS-1), full-length SYS-1; pK-Myc-C3(WRM-1), full-length WRM-1; and pK-Myc-C3(WRM-1AR), WRM-1(135–560), deletion of the 134 N-terminal and of the 236 C-terminal amino acids. The following HA-tagged proteins were expressed from pHAK-B vectors, HA-tag variants of the pK-Myc-C plasmids: pHAK-B2(NHR-25), full-length NHR-25; pHAK-B2(NHR-25ΔL), NHR-25(1–320), deletion of the ligand binding domain; and pHAK-B3(POP-1), full-length wild-type POP-1. Mutant forms POP-1(D9E), corresponding to POP-1(p645) (Siegfried and Kimble, 2002), and POP-1(D9A) were generated by PCR with forward primers, in which the ninth GAT codon had been mutated either to GAA (glutamic acid) or to GCT (alanine) and expressed from pHAK-B3. The pEGFP-C2 plasmid (Clontech) was used for expression of full-length NHR-25 and its truncated version NHR-25ΔL, N-terminally fused with EGFP.

Yeast Two-Hybrid Assay

Yeast two-hybrid analyses were performed by using the Matchmaker Two-Hybrid System 3 (Clontech). Yeast transformation was done with the lithium acetate method as described by the manufacturer. Bait (Gal4 DNA binding domain fusion) and prey (Gal4 activation domain fusion) plasmids in the 2:1 ratio, respectively, were cotransformed in a single step. Transformants were selected on SD/-Leu/-Trp plates after incubation at 30ºC for 3 days. Several single colonies were picked for each transformant, resuspended in 100 μl of water, and cell numbers were counted. Suspensions were diluted about ten and 100 times to equal cell concentrations, and 5 μl aliquots from each dilution were spotted onto SD/-Leu/-Trp and SD/-Ade/-His/-Leu/-Trp plates. Yeast growth was photographed after 3 days of incubation at 30ºC.

GST Pull-Down Assay

GST alone and full-length GST-BAR-1, GST-WRM-1, and GST-SYS-1 fusion proteins were expressed from pET-42b (Novagen) in *E. coli* strain BL21-CodonPlus (Stratagene) and bound to Glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech). The full-length NHR-25 protein was produced from the pHAK-B2(NHR-25) construct in vitro by using the T7 TNT Coupled Reticulocyte System (Promega). Standard 50 μl transcription-translation reactions were supplemented with 4 μl [^{35}S]-methionine (ICN Biomedicals) per reaction. Four microliters of the radiolabeled NHR-25 were incubated with the immobilized GST-fused proteins in 20 mM HEPES (pH 7.9), 150 mM KCl, 10% glycerol, 0.2% Tween-20, 1 mM DTT, Protease Inhibitor Cocktail (Roche) for 1 hr at 4ºC. Beads were collected by centrifugation and washed six times, 10 min per wash, in 20 mM HEPES (pH 7.9), 200 mM KCl, 10% glycerol, 0.2% Tween-20, 1 mM DTT at 4ºC. Bound proteins were separated on 10% SDS-PAGE and analyzed by autoradiography.

Cell Culture, Transfections, and Luciferase Assays

Human embryonic kidney (HEK) cell line 293 (EAAC) was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Hyclone) and antibiotics. Only mycoplasma-free cells were used in the experiments. Transfections were performed with the Lipofectamine reagent (Invitrogen).

Reporter gene assays were performed as described previously (Valenta et al., 2003) with the firefly luciferase pTOPFLASH and pFOPFLASH reporters (Korinek et al., 1997) to assay POP-1/TCF-mediated transcription. The transcriptional activity of NHR-25 was tested with two types of reporters. First, pOLDO-luciferase constructs were driven by two copies of the SF-1/Ftz-F1 binding consensus sequence TCAAGGTCA or by two copies of the ROR binding site TCTAGGTCA, a gift from M. Van Gilst (Fred Hutchinson Cancer Research Center, Seattle, WA) (Van Gilst et al., 2002). Second, a 5×SF-1 luciferase reporter containing five copies of the SF-1 response element from the mouse *CYP21* gene (Ikeda et al., 1993) was provided by G. Hammer (University of Michigan, Ann Arbor, MI).

Cells were seeded onto 12-well plates (approximately 2×10^5 cells per well) and in triplicates were transfected 4 hr later with a Lipofectamine mixture containing 50 ng pTK-*Renilla* plasmid (Promega) as an internal control, 400 ng luciferase reporter plasmid, and up to 3 μg appropriate expression vector(s) (these DNA amounts therefore apply to the whole set of three wells). The total amount of DNA was kept constant by adding empty expression vector where necessary. Twenty-four hours posttransfection, the cells were harvested and processed by using the Dual Luciferase Reporter Assay System (Promega). All reporter gene assays were done in triplicates, and data were presented as average values with standard deviations from a minimum of three independent experiments after normalization against the *Renilla* luciferase activities.

In Situ Hybridization

In situ hybridization of *nhr-25* mRNA was performed on wild-type (N2) animals with a digoxigenin-labeled cDNA probe (clone *yk663g4*) as previously described (Asahina et al., 2000 and http://nematode.lab.nig.ac.jp/method/index.php).

Antibody Staining and Coimmunoprecipitation

Rabbit antiserum against a portion of the ligand binding domain of NHR-25 was described previously (Silhankova et al., 2005). For immunostaining of *C. elegans*, this antiserum was purified on a PROSEP-rA High Capacity recombinant protein A column (Millipore). Larvae were prepared and stained as described (Silhankova et al.,

2005), except that the purified anti-NHR-25 antibody was diluted 1:200, and detection was with 1:500 goat anti-rabbit IgG conjugated with biotin (Molecular Probes), followed by 1:500 streptavidin-Alexa 568 (Molecular Probes). Worms were mounted in the VectaShield (Vector) medium containing 1 μg/ml DAPI for DNA counterstaining. Images were captured with Axioplan 2 Zeiss fluorescent microscope.

Epitope-tagged proteins were detected with anti-Myc 9E10 (Roche), anti-HA 12CA5 (Roche), and anti-GFP JL-8 (Clontech) mouse monoclonals or with rabbit antiserum against bacterially expressed full-length EGFP. For coimmunoprecipitations, 0.5–1.0 × 10^7 cells were harvested, washed twice in ice-cold PBS, and lysed in extraction buffer: 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, 0.5% Triton X-100, 0.2 mM EDTA, 0.75 mM β-mercaptoethanol, and protease inhibitors (Roche) for 10 min on ice. Lysates were briefly sonicated on ice, incubated in a rotator for 15 min at 4ºC, and centrifuged (13,000 rpm, 4ºC, 20 min). The supernatant was transferred to a fresh tube and incubated with the appropriate antibody coupled to protein A Sepharose beads (Pierce) (20 μl rabbit antiserum were preincubated with 25 μl of beads for 1 hr at 4ºC and then washed three times with the extraction buffer). The mixture was incubated in a rotator for 3 hr at 4ºC, centrifuged, the supernatant was removed, and the precipitate was washed four times with 1 ml extraction buffer containing 0.3% Tween-20 (Fluka) instead of Triton X-100. Proteins were boiled in SDS sample buffer, separated on SDS-PAGE, and transferred to polyvinylidene fluoride membranes (Pall Gelman Laboratory). After incubation with the appropriate primary antibodies, proteins were detected with horseradish peroxidase-conjugated goat anti-mouse (BioRad) or goat anti-rabbit (Sigma) polyclonal antisera, followed by visualization with an enhanced chemiluminescence system (Pierce).

Supplemental Data

Supplemental Data include one figure and three tables and are available at http://www.developmentalcell.com/cgi/content/full/11/2/203/DC1/.

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Supplemental Data

Crosstalk between a Nuclear Receptor and β-Catenin Signaling Decides Cell Fates in the *C. elegans* Somatic Gonad

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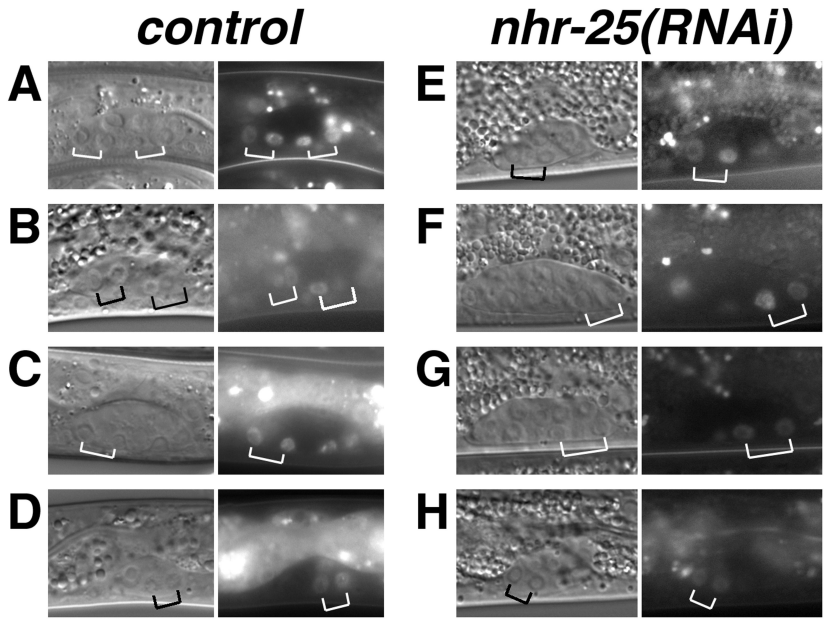

Figure S1. POP-1 Asymmetry in the Somatic Gonad Precursors

Freshly hatched larvae of the JK3437 strain that expresses a GFP::POP-1 fusion protein were fed either control bacteria containing the pPD129.36 empty vector (left panel), or bacteria expressing *nhr-25* dsRNA (right panel). Brackets show pairs of unequally divided daughters of the Z1 and/or Z4 cells. Expected asymmetry of GFP::POP-1 (stronger signal in the nuclei of the proximal daughters) is visible in control (A and B) as well as in RNAi treated L1 worms (E and F). However, in some animals the asymmetry was not apparent regardless whether they were subjected to *nhr-25* RNAi. The experimental cohort of animals showed the typical frequency of the *nhr-25* gonadal defect.

Table S1. *nhr-25* RNAi Affects DTC and AC Numbers Only during Early Gonad Development

		Number of DTCs (% of RNAi treated worms)					**Number of AC (%)**		
	hours[1]	**1**	**2**	**3**	**4**	**n**	**0**	**1**	**n**
empty vector	**0**	4	96	0	0	160	1	99	382
nhr-25(RNAi)		3	47	39	11	168	34	66	465
empty vector	**4**	4	96	0	0	186	1	99	194
nhr-25(RNAi)		3	94	3	0	196	6	94	198
empty vector	**7**	6	94	0	0	105	0	100	77
nhr-25(RNAi)		3	97	0	0	88	2	98	98
empty vector	**10**	7	93	0	0	107	2	98	101
nhr-25(RNAi)		2	98	0	0	124	3	97	102
empty vector	**20**	7	93	0	0	187	1	99	205
nhr-25(RNAi)		5	95	0	0	168	3	97	190

[1]Time of RNAi application since hatching (0 hours). For RNAi applied later than from hatching, newly hatched worms were first fed for 4, 7, 10 or 20 hours, respectively, on control bacteria containing the pPD129.36 empty vector, and then transferred to plates seeded with either the same control bacteria or bacteria expressing *nhr-25* dsRNA.

Table S2**.** *nhr-25* Genetically Interacts with *pop-1* and *sys-1* during Early Gonad Development

		Fertility[3]		**Arm elongation**	
	hours[1]	**%**	**n**	**%**	**n**
pop-1(q645); empty vector[2]	0	0	126	0	28
pop-1(q645); nhr-25(RNAi)	0	16	141	61	44
pop-1(q645); empty vector	10	0	20	0	20
pop-1(q645); nhr-25(RNAi)	10	0	26	0	26
sys-1(q544); empty vector	0	0	30	0	30
sys-1(q544); nhr-25(RNAi)	0	20	30	37	30

[1]Time of RNAi application since hatching (0 hours).
[2]Worms fed on control bacteria containing the empty pPD129.36 vector.
[3]Offspring of fertile *pop-1(q645); nhr-25(RNAi*) and *sys-1(q455); nhr-25(RNAi*) parents all displayed the Sys gonadal defect.

Table S3. Effects of *nhr-25*, *pop-1* and *sys-1* RNAi on DTC Number

RNAi targets[1]	**DTC number (% animals)**		
	missing (0-1)	**extra (3-4)**	***n***
nhr-25 + empty vector[2]	3	35	511
pop-1 + empty vector	44	0	220
sys-1 + empty vector	56	0	245
nhr-25 + *pop-1*	10	22	294
nhr-25 + *sys-1*	42	4	262

[1]For double knockdown experiments, equal amounts of bacteria from two cultures were mixed.
[2]To allow comparisons between double and single knock-down experiments, equal amounts of control bacteria containing the empty pPD129.36 vector were added.

3.4 Signalizace Wnt blokuje apoptózu indukovanou ligandem TRAIL v lidských maligních hematopoietických buňkách

Signalizace Wnt je důležitým kontrolním mechanismem, který ovlivňuje proliferaci a přežívání řady buněčných typů, mimo jiné se uplatňuje při regulaci těchto procesů u pre-B buněk a některých typech chronických leukémií [151, 232, 233]. Proliferační stimulace buněk prostřednictvím signalizace Wnt tak může interferovat s jinými buněčnými mechanismy, které řídí osud buňky, jako například s programovanou buněčnou smrtí - apoptózou. Rozhodnutí o tom, zda buňka přežije, či podstoupí apoptózu, je tedy výsledkem integrace apoptotických a proliferačních stimulů. V této práci jsme se zaměřili na studium vlivu signalizace Wnt na apoptózu vyvolanou ligandem TRAIL u hematopoietických buněk. Ligand TRAIL (Tumor necrosis factor- Related Apoptosis-Inducing Ligand) patří do skupiny ligandů příbuzných k TNF (Tumor Necrosis Factor) a tak jako většina těchto ligandů indukuje u senzitivních buněk apoptózu zprostředkovanou tzv. receptory smrti (death receptors). TRAIL interaguje se specifickými receptory DR4 a DR5, tím indukuje vznik intracelulárního proteinového komplexu DISC (Death-Inducing Signaling Complex) a následnou aktivaci iniciační kaspázy 8 (nebo kaspázy 10). Tato iniciační kaspáza jednak aktivuje další tzv. efektorové (označované rovněž jako exekuční) kaspázy, např kaspázu 3, které iniciují degradaci řady buněčných proteinů. Druhou aktivitou iniciační kaspázy 8 je přeměna neaktivního proapoptotického proteinu Bid (člen proteinové rodiny Bcl-2) v jeho aktivní zkrácenou formu. Aktivní Bid je pak translokován do mitochondrií, kde se podílí na aktivaci mitochondriální apoptotické mašinerie [234-237].

Jak je dokumentováno v předkládané práci, ligand TRAIL indukuje apoptózu u řady hematopoiteckých buněčných linií. Jako základní testovací systém, který by umožnil sledovat vliv signalizace Wnt na apotózu indukovanou ligandem TRAIL, jsme zvolili kokultivaci příslušných hematopoietikých buněčných linií s transformovanými fibrobroblasty Rat2, které exprimují Wnt1 (buňky Rat2Wnt1), respektive kontrolní vektor (buňky Rat2MV7). Kokultivace s buňkami produkujícími Wnt1 vedla u některých buněčných linií k částečné blokaci apoptózy indukované ligandem TRAIL (linie T-buněčného původu Jurkat a CEM). U testovaných pre-B buněčných liniích KM3 a REH vedla kokultivace s buňkami Rat2Wnt1 k takřka kompletní inhibici apoptózy, v případě myeloidních buněk nebyla apoptóza nijak ovlivněna. Hlavním mechanismem, jak signalizace Wnt ovlivňuje apoptózu iniciovanou ligandem TRAIL se zdá být vliv na expresi proapoptotických receptorů DR4 a DR5 v cílových buňkách. Kokultivace

s buňkami exprimujícími Wnt1 vede u pre-B buněčné linie KM3 k inhibici exprese DR4 a DR5 na úrovni mRNA i proteinu. Snížení exprese těchto receptorů na povrchu cílových buněk způsobuje, že ligand TRAIL nenachází na cílových buňkách své vazebné partnery, což vede k inhibici apoptózy (mimo jiné nedochází k aktivaci kaspáz). Kokultivace s buňkami Rat2Wnt1 vede u buněk KM3 rovněž ke stabilizaci β-kateninu a změnám v expresi některých cílových genů signalizace Wnt. Zvyšuje se exprese genu *c-myb* a naopak se snižuje exprese genu *p21* a paradoxně se také snižuje exprese genu *c-myc*. Snížená exprese genu *c-myc* ovšem může být v tomto případě důležitá pro inhibici apoptózy. Jak bylo dříve publikováno c-myc může zvýšit intenzitu apoptózy indukované ligandem TRAIL v případě epiteliální linie HA1E [238]. Snížení exprese c-myc spolu s inhibicí transkripce p21 může tedy působit anti-apoptoticky. Aditivním mechanismem, který přispívá k inhibici apoptózy se zdá být aktivace kinázové dráhy MEK1/ERK1 v cílových buňkách způsobená ligandem Wnt. Některé práce ukázaly, že ligandy Wnt mají za jistých podmínek schopnost aktivovovat zmíněnou kinázovou dráhu [239, 240]. Signální dráha MEK1/ERK1 má schopnost inhibovat apoptózu vyvolanou ligandem TRAIL [241].

Stabilizace β-kateninu způsobená ligandem Wnt ovšem není dostačující podmínkou pro inhibici apoptoptózy vyvolané ligandem TRAIL. Experimenty s využitím rekombinantního proteinu Wnt, nebo Wnt-kondiciovaného média, či jiných buněk produkujících ligand Wnt, naznačují, že pro úplnou inhibici apoptózy je nutná kokultivace s buňkami Rat2Wnt1. Je tedy možné, že pro inhibici apoptózy je kromě samotného ligandu Wnt nutný další extracelulární membránový faktor, který je produkovaný buňkami Rat2Wnt1 jako výsledek jejich autostimulace ligandem Wnt.

Antiapoptotické působení signální dráhy Wnt bylo ukázáno rovněž jinými skupinami. Signální dráha Wnt se podílí na inhibici apoptózy u krysích fibroblastů, osteoblastů a některých střevních buněk [242]. Jak ukázala naše práce, signalizace Wnt může inhibovat apoptózu indukovanou některými ligandy ze skupiny TNF. Vzhledem k tomu, že se seriózně pracuje na využití ligandu TRAIL v rámci protinádorové terapie, je potřeba brát v úvahu při volbě správné terapeutické strategie také možnou interferenci apoptózy indukované ligandem TRAIL a signální dráhy Wnt.

Wnt-induced signaling suppresses TRAIL-mediated apoptosis of human malignant hematopoietic cells

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