

# Wnt/ $\beta$ -catenin signaling regulates the expression of the homeobox gene *Cdx1* in embryonic intestine

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## SUMMARY

During mammalian development, the *Cdx1* homeobox gene exhibits an early period of expression when the embryonic body axis is established, and a later period where expression is restricted to the embryonic intestinal endoderm. *Cdx1* expression is maintained throughout adulthood in the proliferative cell compartment of the continuously renewed intestinal epithelium, the crypts. In this study, we provide evidence in vitro and in vivo that *Cdx1* is a direct transcriptional target of the Wnt/ $\beta$ -catenin signaling pathway. Upon Wnt stimulation, expression of *Cdx1* can be induced in mouse embryonic stem (ES) cells as well as in undifferentiated rat embryonic endoderm.

Tcf4-deficient mouse embryos show abrogation of Cdx1 protein in the small intestinal epithelium, making Tcf4 the likely candidate to transduce Wnt signal in this part of gut. The promoter region of the *Cdx1* gene contains several Tcf-binding motifs, and these bind Tcf/Lef1/ $\beta$ -catenin complexes and mediate  $\beta$ -catenin-dependent transactivation. The transcriptional regulation of the homeobox gene *Cdx1* in the intestinal epithelium by Wnt/ $\beta$ -catenin signaling underlines the importance of this signaling pathway in mammalian endoderm development.

Key words: Transactivation, Wnt, TCF4/ $\beta$ -catenin, Cdx1

## INTRODUCTION

Members of the Wnt family of signaling proteins have diverse and important functions during development in a wide range of multicellular organisms (Cadigan and Nusse, 1997; Moon et al., 1997). Genetic and biochemical studies have identified the factors involved in Wnt signal transduction and have demonstrated that this signaling pathway is evolutionarily highly conserved (Moon and Kimelman, 1998; Gumbiner, 1998; Gradl et al., 1999). A key player in the Wnt signaling cascade is  $\beta$ -catenin or in *Drosophila* its homologue Armadillo (Willert and Nusse, 1998).  $\beta$ -catenin is a multifunctional protein which, in addition to its role in transmitting Wnt signals, is involved in several other cellular processes, such as cell-cell adhesion, linkage of cadherins to the cytoskeleton (Aberle et al., 1996), or interaction with regulatory tyrosine kinase receptors and phosphatases (Huber et al., 1996a). The cytosolic level of  $\beta$ -catenin is tightly regulated; this is assured by its interaction with a multimeric complex composed of glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), Axin/Conductin and the Adenomatous polyposis coli (APC) tumor suppressor protein (Polakis, 1999). Constitutive phosphorylation of cytosolic  $\beta$ -catenin by GSK3 $\beta$  directs  $\beta$ -catenin to the ubiquitin-proteasome degradation pathway (Aberle et al.,

1997; Yost et al., 1996). Wnt signaling negatively regulates the action of GSK3 $\beta$  on  $\beta$ -catenin and thus leads to an increased cytosolic level of the protein.  $\beta$ -catenin, alone or in association with members of the Tcf/Lef1 (T cell factor/Lymphocyte enhancer factor1) family of transcription factors, can translocate to the nucleus, where it ultimately controls the activity of specific target genes (Molenaar et al., 1996; Behrens et al., 1996; Huber et al., 1996b). Although the exact molecular mechanism by which the Tcf/ $\beta$ -catenin-complex regulates transcriptional activity of target genes is at present only beginning to be understood, it is generally accepted that Tcf factors bind to specific DNA sequences, whereas  $\beta$ -catenin confers transactivating function. Direct interaction of  $\beta$ -catenin with components of the basic transcriptional machinery has been shown (Hecht et al., 1999), while several reports underline the importance of additional nuclear factors in modulating the transactivation activity of Tcf/ $\beta$ -catenin (Hecht et al., 2000; Waltzer and Bienz, 1998; Roose et al., 1998; Cavallo et al., 1998). Besides its role in developmental processes, the Wnt signaling pathway has also been implicated in carcinogenesis (Peifer and Polakis 2000). For example, the proto-oncogene *Wnt1* promotes mammary gland hyperplasia in mice (Nusse and Varmus, 1982) and unphysiologically stabilized  $\beta$ -catenin has been observed in colon carcinomas,

hepatocellular carcinomas and melanomas (Berx et al., 1998). In addition, inherited disruption at the APC locus causes familial adenomatous polyposis and loss of APC function is one of the most frequent events in sporadic colorectal cancer (Kinzler and Vogelstein, 1996).

All multicellular organisms require cell communication to regulate growth and differentiation during development. Secreted signaling proteins represent one important mechanism to coordinate the developmental program of cells and tissues. In particular, members of the Wnt family have been implicated in diverse biological processes, such as pattern formation in *Drosophila*, the specification of the vertebrate body axis, neural tube development and morphogenesis of several organs, such as mammary glands, kidneys or teeth (Wodarz and Nusse, 1998). In general, stimulation by Wnt can result in an immediate cell fate decision by regulating target genes that specify the differentiated cell type. Alternatively, Wnt signals can activate secondary signals that are subsequently responsible for the diversification of cell fates. Indeed, examples for both possibilities can be found among the reported direct target genes regulated by Wnt signaling, such as those for the cellular components fibronectin (Gradl et al., 1999) and matrilysin (Brabletz et al., 1999); transcription factors, such as *Drosophila* Ubx (Riese et al., 1997), *Xenopus* Siamois (Brannon et al., 1997) and mammalian Tcf1 (Roose et al., 1999) and Brachyury (Yamaguchi et al., 1999; Arnold et al., 2000); secreted protein nodal-related 3, which is a member of the transforming growth factor beta family (Xnr3; McKendry et al., 1997), but also genes that are involved in cell proliferation and which are upregulated in human colon cancer, including *c-myc* (He et al., 1998), *cyclin D1* (Tetsu and McCormick, 1999; Shtutman et al., 1999) and *c-jun* (Mann et al., 1999).

Homeobox genes encode DNA-binding proteins that play crucial roles during development to define the body plan and to determine cell fate (Krumlauf, 1994; McGinnis and Krumlauf, 1992). For example, the *Drosophila caudal* gene participates in the definition of the anteroposterior axis during early embryogenesis, and its expression is maintained in several organs including gut (Macdonald and Struhl, 1986). *Cdx1* is one of the three *caudal* homologues identified in mammals. Its expression pattern has been analyzed during murine embryonic development by in situ hybridization and by immunohistochemistry (Duprey et al., 1988; Meyer and Gruss, 1993; Subramanian et al., 1998). *Cdx1* expression starts during gastrulation (day 7.5) in ectodermal and mesodermal cells when the anteroposterior axis is established, and it is progressively expressed along the embryonic axis in a rostral-to-caudal direction up to day 12. Lack of *Cdx1* in mice causes homeotic transformation of vertebrae and dysregulation of downstream *Hox* genes, consistent with a role of *Cdx1* in defining positional information along the rostrocaudal axis (Subramanian et al., 1995). Beginning with day 14 of gestation in mice, *Cdx1* is selectively expressed in the endoderm of the developing intestine, where it becomes restricted to and maintained in the proliferative crypt compartment during epithelium differentiation.

In our attempts to identify target genes of the Wnt/ $\beta$ -catenin signaling pathway in early mouse embryonic development, we have used embryonic stem (ES) cells co-cultured on NIH3T3 fibroblasts expressing different Wnts as feeder cells (Arnold et

al., 2000). We report here that the mouse homeobox gene *Cdx1* is a direct target of Wnt/ $\beta$ -catenin signaling.

## MATERIALS AND METHODS

### Plasmid constructions

The reporter plasmid pCdx1-4Luc containing the -3,621 bp promoter fragment of the murine *Cdx1* gene driving a luciferase reporter gene was constructed using pGL3-Basic (Promega), following standard procedures and as described (Lorentz et al., 1999). The deletion constructs pCdx1 $\Delta$ ELuc, pCdx1 $\Delta$ BLuc, pCdx1 $\Delta$ SELuc, pCdx1 $\Delta$ PSLuc and pCdx1 $\Delta$ ANLuc were generated from the pCdx1-4Luc vector by restriction digestion and religation (illustrated in Fig. 2A). For the point mutation of the Tcf/Lef-binding-elements TBE2 (5'-CTTTGTT-3'; at position: -950), TBE3 (5'-CTTTGAT-3'; pos.: -113) and TBE4 (5'-CTTTGAA-3'; pos.: -82) in the pCdx1 $\Delta$ PSLuc plasmid, the underlined bases were replaced by GC. For this, the *KpnI*, *HindIII* restriction fragment containing the -1,441 bp promoter fragment was subcloned into the pBluescriptIIKS<sup>+</sup>-plasmid (Invitrogen), giving plasmid pBKS<sup>+</sup>-Cdx1-1.4, which was used in site-directed mutagenesis of TBE2-4, as mentioned above, using the QuikChange<sup>TM</sup> Site-Directed Mutagenesis Kit (Stratagene). Details of PCR primers can be obtained from the authors (e-mail: lickert@immunbio.mpg.de). The mutated promoter fragment was sequenced and recloned into pCdx1 $\Delta$ PSLuc.

### Cell lines and transfections

HEK293 and NIH3T3 fibroblasts were grown in DMEM with 10% fetal calf serum (FCS). Mouse embryonic stem (ES) cells were maintained on embryonic fibroblast feeder cells or were grown in 1000 units/ml LIF (Gibco) per ml on gelatin-coated tissue culture plates. The co-cultivation of ES cells with NIH3T3 fibroblasts was done as described (Arnold et al., 2000; Kispert et al., 1998).

Transient transfections were carried out using the calcium phosphate precipitation method. HEK293 cells were plated at a density of  $3 \times 10^5$  on 6-well plates one day before transfection. For transient reporter assays 1  $\mu$ g of the reporter-plasmid together with 0.5  $\mu$ g of a pCMV- $\beta$ -galactosidase-expressing plasmid (to monitor transfection efficiency) served as a reporter mix, whereas for normalization of DNA amount 0.25  $\mu$ g pcDNA3-TCF4E-myc (Tetsu et al., 1999), 0.5  $\mu$ g pCS2<sup>+</sup>S33A (Aberle et al., 1997) or 0.5  $\mu$ g pCS2<sup>+</sup> empty vector was used in the indicated combinations as inducer mixes. The  $\beta$ -galactosidase expression was not significantly affected by either Tcf4 or  $\beta$ -catenin cotransfection. For the inhibition experiments 0.5  $\mu$ g of plasmid DNA-pcDNA3- $\Delta$ NTCF4E-myc, pcDNA3-TCF4E-myc, pCS2<sup>+</sup> $\beta$ -catenin (Aberle et al., 1997) or pCS2<sup>+</sup>S33A was cotransfected as indicated in Fig. 2B,C, together with the reporter mix described above. In all reporter assays, the cells were lysed after 48 hours, and luciferase and  $\beta$ -galactosidase activities were determined on an EG&G Berthold Autolumat LB953. Luciferase activity was normalized to  $\beta$ -galactosidase activity as an internal transfection control.

### Northern blot hybridization

Total RNA from cells grown in co-culture for 24 hours was isolated with the RNeasy<sup>TM</sup> B method (TEL-TEST, Inc.), according to the manufacturer's description. RNA preparations were further purified by one or two passages over an oligo(dT) column (mRNA purification kit; Pharmacia). RNA samples were electrophoresed through 1% denaturing agarose gels and transferred to Hybond N<sup>+</sup> membrane (Amersham) by capillary transfer in 10 $\times$  SSC. After transfer, the blot was baked at 80°C for 2 hours and the nucleic acids were visualized by methylene blue staining. The blots were hybridized with the 1.6 kb *HindIII* murine *Cdx1* cDNA from the pCdx1-S vector (Lorentz et al., 1997) and as a loading control hybridized with a 500 bp PCR-product of GAPDH (forward GAPDH: 5'-ACCACAGTCTGCCA-

TCAC-3'; reverse GAPDH: 5'-TCCACCACCCTGTTGCTGTA-3'). The probes were radioactively labeled with 50  $\mu$ Ci [ $\alpha$ - $^{32}$ P]dCTP using the Megaprime<sup>TM</sup> DNA labeling system (Amersham Pharmacia Biotech). The filter was hybridized overnight at 65°C in a solution containing 4 $\times$  SSC, 1% SDS, 1% non-fat milk powder, 20% dextran sulfate, 0.5 mg/ml salmon sperm DNA and approx.  $10^7$  cts/minute labeled probe. After hybridization, the membrane was washed in 2 $\times$  SSC, 0.1% SDS for 15 minutes at 60°C and then twice in 0.1% SSC, 0.1% SDS at 60°C for 30 minutes each. The filter was then exposed to film (Kodak BMR) with an intensifying screen (DuPont Lightning Plus) at -70°C.

#### Antibodies, western blot analysis and affinity precipitation

The mouse monoclonal antibody against  $\beta$ -catenin was obtained from Transduction Laboratories (Lexington, KY). The rabbit anti-*Cdx1* polyclonal antibody was raised against an amino-terminal peptide of the mouse *Cdx1* protein (Meyer et al., 1993). Cell lysates from  $5\times 10^5$  transiently transfected HEK293 cells (2  $\mu$ g p*Cdx1*-S) or from co-cultured ES cells were prepared and 100  $\mu$ g total protein was loaded on a 10% PAA-gel and separated by SDS-PAGE. Western blotting was carried out as described previously (Aberle et al., 1997).

#### Electrophoretic mobility shift analysis

Recombinant GST-Lef1 aa 1-397 and  $\beta$ -catenin-His<sub>6</sub> proteins were prepared as described (Arnold et al., 2000). The *Cdx1* promoter fragments spanning the TBE3 and TBE4 binding motifs were amplified in a PCR reaction in the presence of 5  $\mu$ Ci [ $\alpha$ - $^{32}$ P]dCTP with primers covering the wild-type or mutated sites (fwd-TBE3wt: 5'-GGGCTTCCCCCTTTGATTTCGCGGCCCC-3', rev-TBE4wt: 5'-CGGCTTTGCATTTCAAAGCGGGGGAA-3'). In the mutations, the underlined bases were replaced by GC. 50 ng of one of the corresponding TBE3 and/or TBE4 wild-type or mutated luciferase reporter plasmids served as a template. The resulting 67 bp fragment was purified over a 2% agarose gel.  $10^4$  cts/minute of these labeled oligonucleotides was incubated in the presence of 20 mM Hepes, pH 7.9; 60 mM KCl; 1 mM EDTA, pH 8.0; 1 mM DTT, 5 mM MgCl<sub>2</sub>, 10% (v/v) glycerol and 1  $\mu$ g poly(dI-dC) in a total volume of 15  $\mu$ l, together with 300 ng GST-Lef1 aa 1-397 protein and an equimolar amount of  $\beta$ -catenin-His<sub>6</sub>, for 30 minutes on ice. To the Lef1/ $\beta$ -catenin DNA complexes was added 1  $\mu$ g of the mouse monoclonal antibody against  $\beta$ -catenin (see above). Electrophoresis was performed through 4% native polyacrylamide gels in 0.25 $\times$  TBE at room temperature.

#### Preparation and analysis of rat embryonic endoderm

Wistar rat fetuses were removed by Caesarian section at day 13 of gestation. The small intestines were isolated and processed as previously described (Duluc et al., 1997). In brief, the endoderm was separated from the mesenchyme with 0.03% collagenase (Roche Boehringer) for 1 hour followed by mechanical dissociation. Each endoderm was cut into small pieces of about 1 mm in length and seeded on 3T3-*lacZ* or 3T3-Wnt1 cells. Endoderm fragments corresponding to five fetal small intestines were co-cultured in 3 cm dishes in 45% Ham-F12, 45% DMEM and 10% fetal calf serum for 24 hours at 37°C.

To amplify the rat *Cdx1* messenger RNA (Duluc et al., 1997), the following primers were used: forward: 5'-GTAAGACTCGGACCAAGGACAAGTA-3'; reverse: 5'-AACTGTGTGGGAGGCATGGGCTGCG-3'. For the amplification of *cytokeratin19* messenger RNA the oligonucleotides were: forward: 5'-TTGAGATTGAGCTGCAGTCC-CAGCT-3'; reverse: 5'-TTCCCAGGGGAGTCTCGCTGGTAGC-3'. The rat *Cdx1* primers do not cross-hybridize with murine *Cdx1* mRNA and there is no detectable expression of *cytokeratin19* mRNA in 3T3 cells.

RNA was prepared from rat endoderm tissue slices using TRI-reagent (Euromedex) according to the recommendations of the manufacturer. RT-PCR was conducted under standard conditions

(Lorentz et al., 1997). Single-stranded cDNA was synthesized at 42°C for 1 hour using 3  $\mu$ g RNA, 25 pmoles oligo(dT) or 25 pmoles CDX1b, in 25  $\mu$ l containing 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM spermidine, 4 mM sodium pyrophosphate, 0.2 mM each dNTP, and 15 units AMV reverse transcriptase (Promega). For PCR, 2  $\mu$ l of single-stranded cDNA was used in 100  $\mu$ l containing 25 pmoles of each primer, 1.25 mM MgCl<sub>2</sub>; 10 mM Tris-HCl, pH 8.8; 50 mM KCl, 0.1% Triton X-100; 0.6 units DyNAzyme DNA polymerase (Finnzymes). Cycles were: 30 seconds at 94°C, 30 seconds at 50°C and 45 seconds at 72°C. PCR was performed for an increasing number of cycles up to 40 cycles. After electrophoresis on 3% agarose gels, PCR fragments were analyzed using an Imaging Densitometer (GelDoc 1000, Bio-Rad).

#### Immunohistochemistry

Embryos were genotyped for the mutation of the *Tcf4* gene according to Korinek et al. (1998b) and were snap-frozen in liquid nitrogen. Sections of 7  $\mu$ m were blocked for endogenous peroxidase activity with 0.3% H<sub>2</sub>O<sub>2</sub> and 0.01% HCl in methanol for 30 minutes, washed in PBS and incubated overnight with a 1:100 diluted of anti-*Cdx1* antibody in 4% normal human serum in a humidified chamber at 4°C (Subramanian et al., 1998). Subsequently, sections were washed and incubated with multiple peroxidase-conjugated anti-rabbit antibodies (Envision, DAKO). The color reaction was developed in buffer containing 3,3'-diaminobenzidine-tetrachloride (DAB, Sigma) with 0.01% H<sub>2</sub>O<sub>2</sub>. Nuclei were counterstained with Hematoxylin.

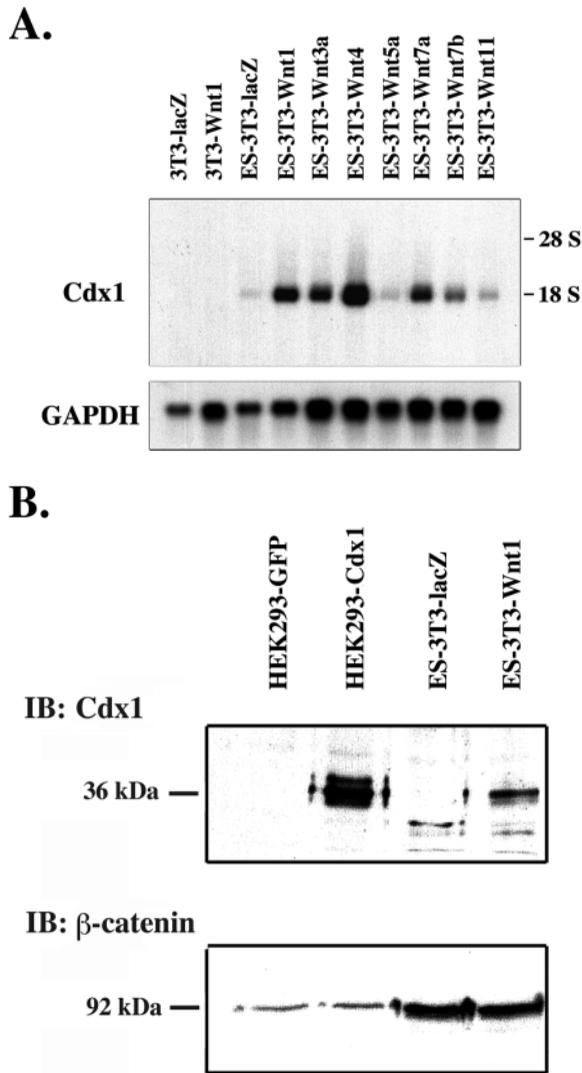
## RESULTS

### Expression of *Cdx1* in Wnt-stimulated ES cells

Preliminary experiments using the chromatin immunoprecipitation method (ChIP) with anti- $\beta$ -catenin antibodies on nuclear extracts of Wnt-expressing fibroblasts and Wnt-induced ES cells indicated that *Cdx1* is a potential target gene of Wnt/ $\beta$ -catenin (not shown). More detailed analysis monitoring *Cdx1* expression in ES cells that had been co-cultured on 3T3 cells expressing Wnt was then applied. Northern blot analysis revealed that several Wnts stimulated *Cdx1* expression in ES cells (Fig. 1A). The strongest expression of *Cdx1* was observed upon Wnt4 stimulation. Wnt1, Wnt3a, and Wnt7a also induced *Cdx1* nearly as well, while Wnt5a and Wnt11 had only little or no effect on *Cdx1* expression and were comparable to the control co-cultured with *lacZ*-expressing 3T3 cells. Induction of *Cdx1* does not require direct cell-cell contact between ES cells and Wnt-expressing 3T3 cells, since induction was also observed when ES cells were seeded on transwell filters placed on top of the inducing 3T3 cells (not shown). Wnts not only induced *Cdx1* mRNA in ES cells, but also *Cdx1* protein synthesis as monitored with anti-*Cdx1* antibodies in immunoblots on cell lysates of ES cells co-cultured on either Wnt1- or *lacZ*-expressing 3T3 cells (Fig. 1B). To control the specificity of anti-*Cdx1* antibodies human HEK293 cells expressing either mouse *Cdx1* or green fluorescent protein (GFP) driven by the CMV promoter were included. These experiments demonstrate that, upon Wnt stimulation, ES cells express *Cdx1* mRNA and *Cdx1* protein.

### Tcf/ $\beta$ -catenin binds and activates a *Cdx1* promoter fragment

Because of the Wnt-dependent induction of *Cdx1* mRNA in ES



**Fig. 1.** Wnts induce *Cdx1* mRNA and protein in ES cells. (A) ES cells were co-cultured on Wnt-expressing 3T3 fibroblasts for 24 hours and poly(A)<sup>+</sup> RNA (2  $\mu$ g per lane) was probed with a 1.6 kb *Cdx1* cDNA by northern blot analysis. Controls included a 0.5 kb *GAPDH* cDNA probe to measure the amount of loaded mRNA, ES cells co-cultured on *lacZ*-expressing 3T3 cells, and Wnt1 and *lacZ*-expressing 3T3 cells. Strong induction of *Cdx1* mRNA was observed when ES cells were stimulated by Wnt4, Wnt1 or Wnt3a. (B) Western blot analysis with anti-*Cdx1* antibodies on ES cell lysates demonstrated the presence of *Cdx1* protein in ES cells co-cultured on Wnt1-expressing 3T3 cells, but not in ES cells cultured on control *lacZ*-expressing 3T3 cells. To control for the specificity of the antibody, HEK293 cells transiently transfected with cDNAs coding for *Cdx1* and green fluorescent protein (GFP) were included. The amount of cell lysates loaded (100  $\mu$ g per lane) was monitored with monoclonal anti- $\beta$ -catenin antibody.

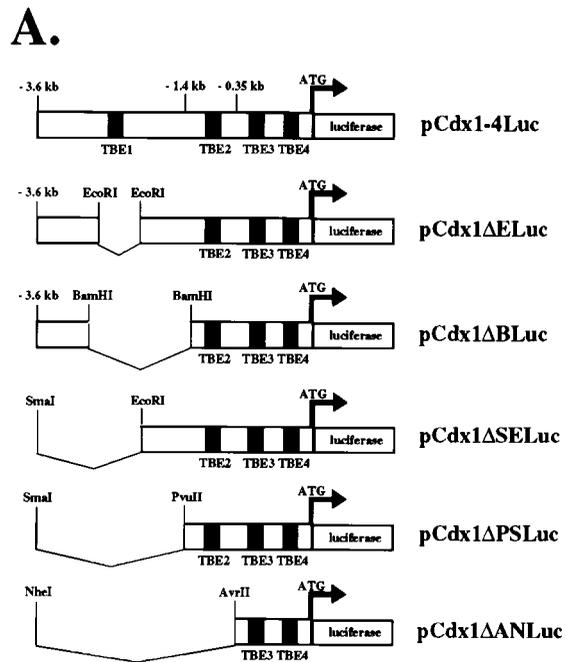
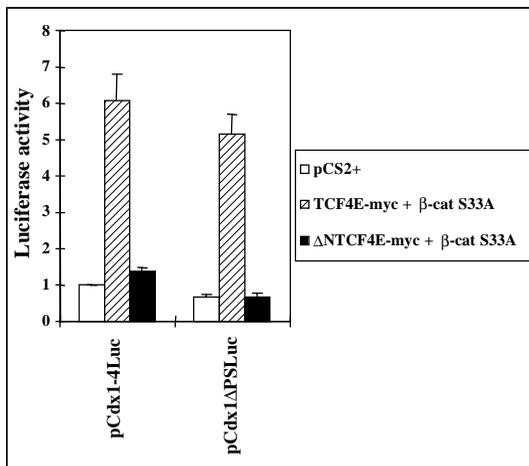
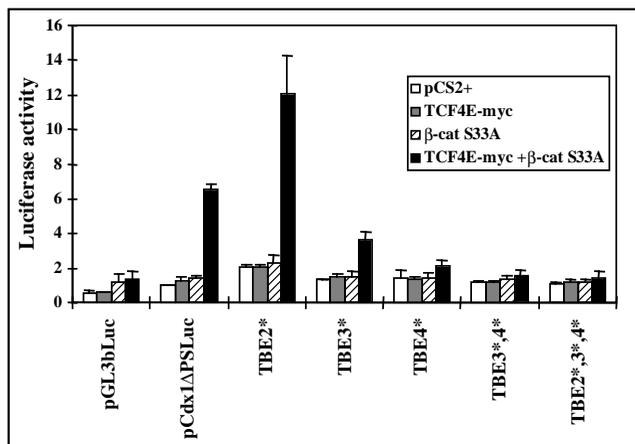
cells, a sequence analysis of the *Cdx1* promoter was made for potential Tcf/Lef1 binding motifs. Four perfect Tcf/Lef1 binding motifs, CTTTGA(T)A(T), were found at positions -1923, -950, -113 and -82 in the *Cdx1* promoter region (numbering relative to the transcription start site; not shown), hereafter numbered TBE1-TBE4 (Tcf-binding element 1-4, see Fig. 2A).

To demonstrate that the *Cdx1* promoter can be regulated by Tcf/Lef1 and  $\beta$ -catenin, luciferase reporter assays were performed in HEK293 cells. A 3.6 kb *Cdx1* promoter fragment harboring TBE sites 1-4 and several deletion constructs thereof were tested (Fig. 2A). All promoter constructs depicted in Fig. 2A exhibited a roughly 6-fold increase of specific Tcf4/ $\beta$ -catenin-mediated activity (not shown). Promoter construct pCdx1-4Luc containing TBE sites 1-4 and construct pCdx1 $\Delta$ PSLuc harboring TBE sites 2-4 conferred comparable yields of  $\beta$ -catenin-dependent activities (Fig. 2B), suggesting that TBE1 is not important for the activation of the *Cdx1* promoter by Tcf/ $\beta$ -catenin in these assays. Controls included plasmid pCS2+ and a dominant-negative version coding for a Tcf4 unable to interact with  $\beta$ -catenin. To analyze in more detail the participation of the *Cdx1* TBE2-TBE4 motifs in the Tcf/ $\beta$ -catenin-mediated transactivation, these sites were mutated individually and in combination (Fig. 2C). The single mutation of TBE4 caused the strongest reduction of  $\beta$ -catenin-dependent activation, and mutating TBE3 considerably reduced the activity, while mutation of TBE2 had no effect or even resulted in a slight increase of  $\beta$ -catenin-dependent activation. The combination of TBE3 and TBE4 mutations abolished the transactivating effect of  $\beta$ -catenin/Tcf4. These experiments demonstrate that the *Cdx1* promoter fragment is regulated by Tcf/ $\beta$ -catenin and that the Tcf-binding sites TBE3 and TBE4 are of major importance.

To assay directly in vitro for binding of Tcf/Lef1 and  $\beta$ -catenin to TBE3 and TBE4, electrophoretic gel-mobility shift analysis was performed with recombinant Lef1 and  $\beta$ -catenin proteins produced in *E. coli* (Fig. 3). A 67 bp fragment of the *Cdx1* promoter comprising the TBE3 and TBE4 binding motifs (see Fig. 2A) was tested for binding of recombinant Lef1 and  $\beta$ -catenin. Lef1 bound specifically to the promoter fragment of *Cdx1*, and co-incubation with  $\beta$ -catenin produced a Lef1/ $\beta$ -catenin complex that interacts with DNA. The size of this complex was further increased by the addition of monoclonal anti- $\beta$ -catenin antibody (Fig. 3A). Mutations in either TBE3 or TBE4, or in both sites together, inhibited binding of Lef1 to DNA (Fig. 3B). In addition, the binding was inhibited in competition experiments by the addition of a molar excess of DNA from double-stranded wild-type probe, but not by adding DNA of the TBE3, TBE4 double mutant form (not shown). These experiments demonstrate specific binding of the Lef1/ $\beta$ -catenin complex to at least two Tcf/Lef1 recognition sites (TBE3 and TBE4) in the *Cdx1* promoter. Taken together the reporter assays and the electrophoretic gel mobility shifts provide strong evidence that *Cdx1* is a direct target gene of the Wnt/ $\beta$ -catenin signaling pathway.

### Wnt signaling stimulates *Cdx1* expression in embryonic endoderm

By day 14 of gestation in mice, *Cdx1* expression turns on and becomes restricted to the intestinal epithelium (Duprey et al., 1988; Subramanian et al., 1998). To examine whether *Cdx1* can be stimulated by Wnt signaling in the developing intestinal endoderm, as was the case in mouse ES cells, small intestinal endoderm from 13-day rat embryos was separated from the mesenchyme and seeded on top of control or Wnt1-expressing 3T3 cells. After 24 hours of co-culture, RNA was extracted for RT-PCR analysis, and the endodermal origin of the RNA was monitored using specific primers for cytokeratin19 transcripts

**B.****C.**

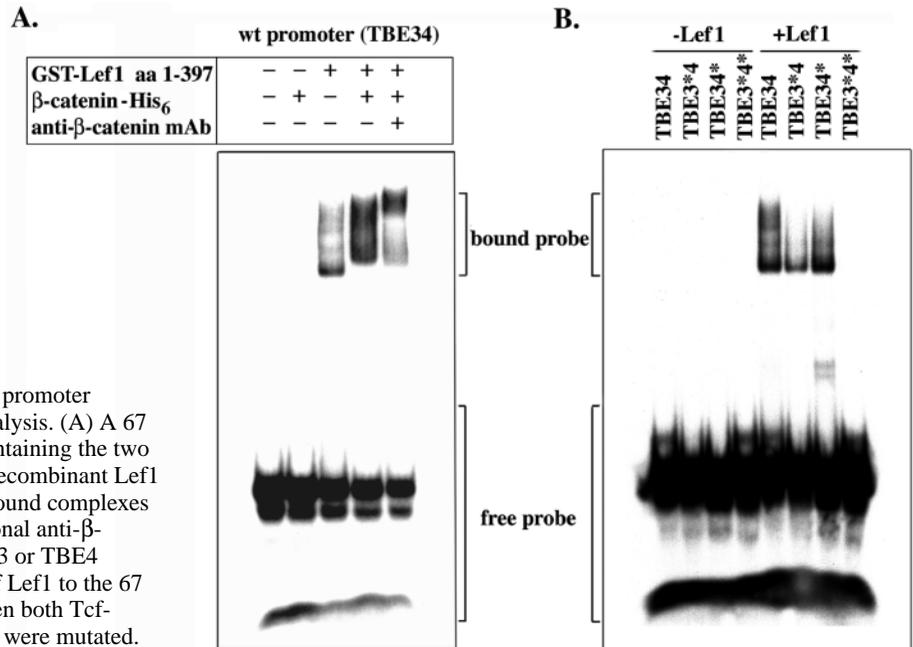
**Fig. 2.**  $\beta$ -catenin-dependent transcriptional transactivation of *Cdx1* promoter fragments in luciferase reporter assays. (A) Schematic representation of the different *Cdx1* promoter fragments used in the reporter assays. The four Tcf-binding motifs in the promoter region, TBE1-TBE4, are depicted as black boxes. (B) Tcf4/ $\beta$ -catenin-dependent transactivation of the *Cdx1* promoter in HEK293 cells. The -3.6 kb and -1.4 kb promoter fragments gave comparable activity, indicating that Tcf-binding motif TBE1 is not essential in these assays. (C) Mutational analysis of TBE2-TBE4 for TCF4/ $\beta$ -catenin-dependent transactivation of the -1.4 kb *Cdx1* promoter fragment. Single mutations in either TBE3 or TBE4 reduced the  $\beta$ -catenin-dependent transactivation, while single mutations in TBE2 gave a slight increase of activity. Mutations in both TBE3 and TBE4 abolished the transactivating effect of  $\beta$ -catenin/TCF4.

(Calnek and Quaroni, 1993). As shown in Fig. 4, rat small intestinal endoderm co-cultured with 3T3-*lacZ* control cells exhibited some weak *Cdx1* expression. In contrast, increased expression of *Cdx1* was observed in co-cultures with 3T3-Wnt1 cells. These results indicate that intestinal endoderm, taken at a stage where it is still undifferentiated, is able to respond to Wnt signal.

#### Lack of Tcf4 abrogates the expression of *Cdx1*

The finding that *Cdx1* expression can be induced in the embryonic endoderm by Wnt1 opened the possibility that, in vivo, Wnt/ $\beta$ -catenin signaling regulates this homeobox gene in the developing intestine. In comparing the expression pattern of *Cdx1* protein with those of components of the Wnt signaling pathway, a very similar expression pattern of *Cdx1* and *Tcf4* and *Tcf3* is notable (Korinek et al., 1998a; James and Kazenwadel, 1991). Specifically, *Cdx1* and *Tcf4* are co-expressed in the proliferative crypt compartment in the intestinal epithelium (Korinek et al., 1998b; Subramanian et al., 1998). Consequently, expression of *Cdx1* in mouse embryos that lack Tcf4 was investigated. Mice deficient for Tcf4 (*Tcf4*<sup>-/-</sup>) exhibit a highly specific phenotype in the small intestine (Korinek et al., 1998b). Mutant embryos show no notable alterations in the organization of the pseudostratified epithelium of the small intestine at E14.5, with an apparently normal transition of intestinal endoderm into epithelium. But at E16.5, *Tcf4*<sup>-/-</sup> embryos lack proliferative cells in the prospective crypt region of the small intestine. If Tcf4 is a candidate to transduce Wnt-dependent expression of *Cdx1*, the expression of *Cdx1* should be affected in the small intestine of *Tcf4*<sup>-/-</sup> embryos.

Hence, immunohistochemistry with anti-Cdx1 antibodies was performed on sections of intestine from *Tcf4*<sup>-/-</sup> embryos or from *Tcf4* heterozygous (*Tcf4*<sup>+/-</sup>) and wild-type (*wt*) littermates for comparison. Anti-Cdx1 antibodies stained the cytoplasm and nuclei of the multilayered intestinal epithelium at E15.5 and strongly stained the nuclei of cells of the proliferative crypt compartment at E17.0 in *wt* (not shown) and *Tcf4*<sup>+/-</sup> littermates (Fig. 5), all in agreement with earlier observations (Subramanian et al., 1998). In contrast, *Tcf4*<sup>-/-</sup> small intestine already exhibited a greatly reduced staining with anti-Cdx1 antibodies at E15.5 when the *Tcf4*<sup>-/-</sup> phenotype is not yet fully apparent (Fig. 5), and no Cdx1 was detected in the prospective crypt region of the small intestine at E17.0 when the mutant phenotype is clearly visible (Fig. 5). However, colon epithelium of *Tcf4*<sup>-/-</sup> embryos was positive for Cdx1, similar to the controls (not shown). From these results, Tcf4



**Fig. 3.** Lef1/ $\beta$ -catenin complexes bind to a *Cdx1* promoter fragment in electrophoretic gel-mobility shift analysis. (A) A 67 bp nucleotide fragment of the *Cdx1* promoter containing the two Tcf-binding motifs, TBE3 and TBE4, bound to recombinant Lef1 and Lef1/ $\beta$ -catenin complexes. The size of the bound complexes was further increased by the addition of monoclonal anti- $\beta$ -catenin antibody. (B) Mutation (\*) in either TBE3 or TBE4 resulted in a reduced binding of Lef1. Binding of Lef1 to the 67 bp oligonucleotide was completely inhibited when both Tcf-binding motifs in the sequence (TBE3\*, TBE4\*) were mutated.

appears to regulate the expression of *Cdx1* in the small intestine.

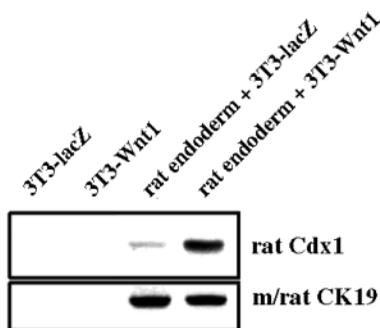
## DISCUSSION

We show here that the mouse homeobox gene *Cdx1* is directly regulated by the Wnt signaling pathway. We have identified four Tcf-binding motifs in the promoter of the *Cdx1* gene and have shown that two of these actually confer Tcf/ $\beta$ -catenin-mediated transcriptional transactivation. Upon Wnt stimulation, *Cdx1* mRNA and Cdx1 protein are induced in embryonic stem (ES) cells. In fact, several Wnts are able to induce *Cdx1* in ES cells co-cultured on Wnt-expressing 3T3 cells. This is likely due to the fact that ES cells express several

different Frizzled receptors and can thus process multiple Wnt signals. We have previously shown that the expression of mouse *Brachyury* can be followed by using this co-culture system (Arnold et al., 2000), and we and others (Yamaguchi et al., 1999) have identified *Brachyury* as a direct target of Wnt signaling. ES cells cultured on Wnt-expressing fibroblasts thus seem well suited for use to identify additional target genes induced by Wnt signals.

## Wnt induces expression of *Cdx1* in the embryonic gut

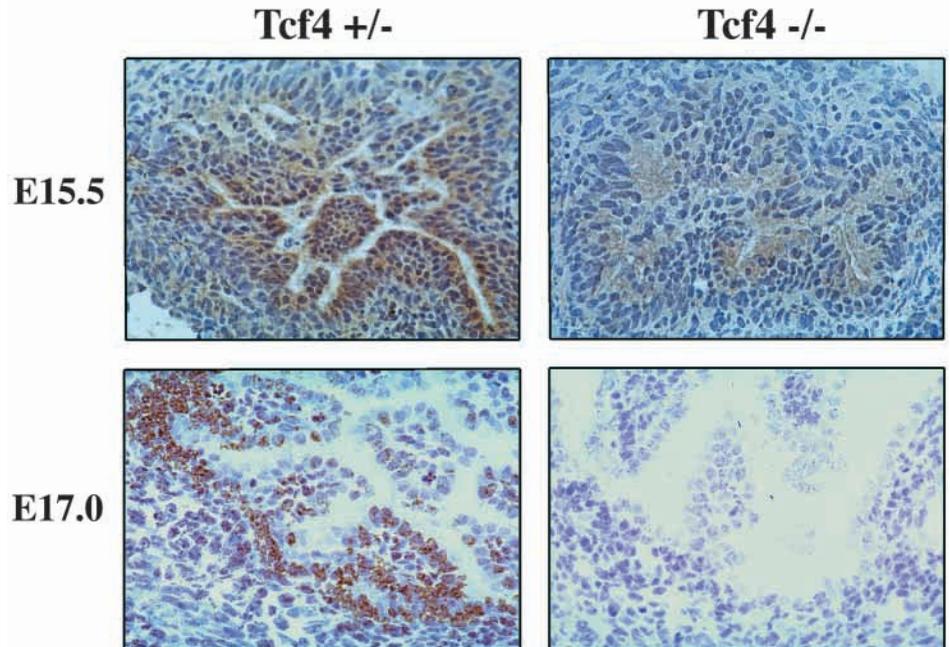
*Cdx1* mRNA and protein have been analyzed during embryonic development and two major regions of expression have been described (Duprey et al., 1988; Meyer and Gruss, 1993; Subramanian et al., 1998). During embryonic axis formation around day 7.5 of gestation, *Cdx1* is expressed in ectodermal and mesodermal cells of the primitive streak. With the regression of the primitive streak, *Cdx1* exhibits a rostral-to-caudal concentration gradient with an anterior expression limit in the prospective hindbrain. At later stages, *Cdx1* expression regresses in its anterior domain and fades out by day 12 pc. At present, we do not know whether the early expression of *Cdx1* is also regulated by Wnt signaling, but our results with ES cells suggest that this is indeed the case. Wnt3a induces *Cdx1* in ES cells and seems a good candidate to regulate *Cdx1* expression during primitive streak formation. The second region of *Cdx1* expression, which we have concentrated on here, is confined to the developing intestinal epithelium. In isolated rat embryonic endoderm taken at day 13 of gestation (E13) when it is still undifferentiated, endogenous *Cdx1* expression can be induced by co-culturing on Wnt1-expressing 3T3 cells. This result, together with the abrogated expression of *Cdx1* in *Tcf4*<sup>-/-</sup> embryos provides convincing evidence that the Wnt signaling pathway is an important regulator of *Cdx1* in the developing endoderm. At present, it is not known which of the Wnt family members are expressed during endoderm development. In preliminary



**Fig. 4.** Induction of *Cdx1* mRNA in embryonic endoderm co-cultured on Wnt1-expressing 3T3 cells. Rat embryonic endoderm from E13 embryos was isolated as described in Materials and Methods, cut into 1 mm pieces and co-cultured on either Wnt1 or *lacZ*-expressing 3T3 cells for 24 hours. Total RNA was isolated and cDNA was made to use as a template in a RT-PCR reaction with primer pairs specific for rat *Cdx1* and *cytokeratin19* (*ck19*) as a control. A clear induction of *Cdx1* expression was observed upon stimulation with Wnt1.

**Fig. 5.** Tcf4 is required for the expression of *Cdx1* in the small intestine.

Immunohistochemistry was performed with anti-*Cdx1* antibodies on cryostat sections of E15.5 and E17.0 embryonic intestine from mice genetically altered for Tcf4. In control embryos heterozygous for Tcf4 (*Tcf4*<sup>+/-</sup>), *Cdx1* was localized in the cytoplasm and nuclei of the pseudostratified epithelium at E15.5 and became highly enriched and localized in the nuclei of cells of the prospective crypt region at E17. Identical results were obtained with wild-type embryos (not shown). In embryos lacking Tcf4 (*Tcf4*<sup>-/-</sup>), *Cdx1* was barely detectable at E15.5 when the *Tcf4*<sup>-/-</sup> phenotype is not fully apparent. No *Cdx1* was detected in E17.0 intestine of mutant embryos. Epithelium of the colon from *wt*, *Tcf4*<sup>+/-</sup> and *Tcf4*<sup>-/-</sup> embryos stained equally well for *Cdx1* (not shown). Sections were counterstained with Hematoxylin. Magnifications: E15.5,  $\times 50$ ; E17.0,  $\times 100$ .



experiments, we have identified several Wnts in embryonic endoderm and in the surrounding mesenchyme (unpublished data). Identification of the physiological Wnt regulator(s) for expression of *Cdx1* will require a more detailed analysis.

#### Tcf4 and endoderm-specific *Cdx1* expression

During days 14-18 of gestation, the mouse intestinal endoderm undergoes cytodifferentiation from a stratified epithelium to form a single columnar epithelium overlying nascent villi and a prospective crypt region between the villi (Mathan et al., 1976). From day E16.5 onwards, the villi are lined by non-cycling, differentiated epithelial cells, whereas the intervilli region, which later forms the crypts, is composed of proliferative and less-differentiated cells (Calvert and Pothier, 1990). *Cdx1* protein becomes detectable in the columnar stages, where it is highly localized in the nuclei of proliferative cells in the prospective crypt region. Thus, *Cdx1* protein is localized predominantly in cells that require functional Tcf4 (Korinek et al., 1998b). Our results with the *Tcf4*<sup>-/-</sup> embryos provide a strong argument that Tcf4 regulates the expression of *Cdx1* in the prospective crypt region of the small intestine. The residual staining of mutant intestine observed at E15.5 might represent background binding by the anti-*Cdx1* antiserum. Alternative explanations are that Tcf4 might not be required for the onset of *Cdx1* expression during early intestinal morphogenesis and/or the presence of another functionally redundant member of the Tcf family here. Tcf3, which exhibits a similar expression pattern to Tcf4 in the embryonic endoderm, could possibly substitute functionally to some extent for the lack of Tcf4 in the small intestine. Such a view is supported by the unchanged expression of *Cdx1* in the colon epithelium of *Tcf4*<sup>-/-</sup> embryos, a region of the embryonic endoderm that expresses a high level of *Tcf3* mRNA and which is not affected by the lack of Tcf4 (Korinek et al., 1998b). If this is the case, our results point to an interesting combinatorial regulation of *Cdx1* expression along the anteroposterior axis of the embryonic endoderm with Tcf4 being responsible for *Cdx1*

expression in the crypt cell compartment of the small intestine and Tcf3 for expression of *Cdx1* in the epithelium of the colon. Taking into account our preliminary findings about the expression of several Wnts in the embryonic endoderm, one can envisage a higher order of combinatorial interactions between Wnts and Tcfs in regulating *Cdx1*. If Wnts are distributed in a regional-specific pattern along the anteroposterior axis of the endoderm and are involved in the determination of cell fates, other Wnt target genes are likely to be discovered that could play a role in the regional specification during endoderm development.

#### Wnt signaling and *Cdx1* in normal and pathological intestine

The *Drosophila* caudal protein is the prototype of the Cdx family of homeodomain transcription factors (Freund et al., 1998). In *Drosophila*, no evidence has been provided so far that *caudal* is regulated by Wingless, but Wingless participates in cell specification along the length of the intestine by regulating the homeobox gene *labial* (Hoppler and Bienz, 1995). A comprehensive description of the role of *Cdx1* in the mammalian intestine is hindered by the fact that no intestinal phenotype has been described so far in *Cdx1*-deficient mice (Subramanian et al., 1995). Likely, *Cdx2*, which is also expressed in the intestinal epithelium, can functionally substitute for the lack of *Cdx1* in the intestine. While this is possible, *Cdx1* and *Cdx2* may instead be regulated differently, since no obvious changes in *Cdx2* mRNA expression were observed in ES cells or endoderm explants co-cultured on Wnt1-expressing 3T3 cells (unpublished data). Evidence obtained in *Drosophila*, *C. elegans*, *Xenopus*, chick and mouse indicates that caudal-related homeodomain proteins control the expression of *Hox* genes in developmental processes. Members of the *Hox* gene family exhibit specific patterns along the length of the murine intestinal epithelium (James and Kazenwadel, 1991), and it has recently been proposed that *Cdx* also controls *Hox* gene expression during endoderm

development (Grapin-Botton and Melton 2000). The results reported in this study indicate a connection between the Wnt pathway and downstream homeobox genes, which may be a common and evolutionarily conserved scheme in endodermal organs.

Several reports have investigated the role of the *Cdx1* and *Cdx2* genes in intestinal cancer. Mice heterozygous for *Cdx2* develop intestinal tumors beside other malformations (Chawengsaksophak et al., 1997) and a recent report suggests a link in humans between *Cdx2* and components of the Wnt signaling pathway in a colorectal cancer cell line (da Costa et al., 1999). The notion derived from these findings is that *Cdx2* might exhibit tumor suppressor activity. The involvement of *Cdx1* in intestinal cancer is less well understood. On the one hand, loss of *Cdx1* expression is observed in a great number of colonic adenocarcinomas (Vider et al., 1997; Silberg et al., 1997). On the other hand, *Cdx1* overexpression stimulates cell proliferation and prevents apoptosis in a rat crypt cell line (Soubeyran et al., 1999), suggesting that *Cdx1* may have a pro-oncogenic potential. This is further suggested by the fact that ectopic *Cdx1* expression is observed in intestinal metaplasia related to Barrett's esophagus, gastric adenomas and liver cholangiocarcinomas (Silberg et al., 1997; Ren et al., 2000) and that *Cdx1* is upregulated by ras activation in a colonic cancer cell line (Lorentz et al., 1999). Our results that Wnt/ $\beta$ -catenin signaling directly regulates murine *Cdx1* provide further support that *Cdx1* is involved in carcinogenesis in digestive organs when this signaling pathway is pathologically activated.

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