Cdx1 Inhibits Human Colon Cancer Cell Proliferation by Reducing β-Catenin/T-cell Factor Transcriptional Activity*

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Rong-Jun Guo‡, Edward Huang‡, Toshihiko Ezaki‡, Neesha Patel‡, Kristen Sinclair§, Jinling Wu§, Peter Klein§, Eun-Ran Suh‡, and John P. Lynch‡‡

From the Divisions of 3Gastroenterology and 5Hematology and Oncology, the Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

Colon cancer is a major cause of cancer morbidity and mortality worldwide. In the United States, over 150,000 people are newly diagnosed with this disease each year, and a third of them will ultimately die from their disease. Many investigations over the years have determined the sequence by which most colorectal cancers arise from normal epithelium. It involves the progression along the adenoma-carcinoma sequence (1, 2). Colorectal cancers arise from adenomas, which are dysplastic but nonmalignant precursor lesions in the colon. Progression along this pathway to cancer occurs through the accumulation of multiple somatic mutations, ultimately leading to malignant transformation and the formation of an invasive colon cancer.

One of the most critical genes mutated in the progression to colorectal cancer is the adenomatosis polyposis coli (APC)1 tumor suppressor. APC mutations occur early and with great frequency in sporadic colon cancer. Familial Adenomatous Polyposis, as well as the Min mouse phenotype, are due to germ line transmission of a mutant APC allele (3). APC has many proposed functions, including the regulation of the Wingless (Wnt) signaling pathway (4–7). β-Catenin is an important intracellular mediator of the Wnt signal. In the unstimulated state, free β-catenin is rapidly recruited from the cytoplasm and the nucleus by APC into a multiprotein complex, actively phosphorylated, and targeted for destruction via the ubiquitin/proteosome pathway (5, 6, 8, 9). This reduces β-catenin levels overall and effectively blocks the Wnt signaling cascade.

When a secreted Wnt protein binds its receptor, Frizzled (Frz) (4–7), the multiprotein complex breaks down and β-catenin accumulates in the cytoplasm. The β-catenin protein then translocates to the nucleus where it complexes with T-cell factor (TCF) and lymphoid-enhancing factors (LEF) to enhance transcription of the Wingless (Wnt) signaling pathway (4–7). β-Catenin is a key regulator of the Wnt pathway (10). APC mutations, in combination with β-catenin mutations, activate the Wnt pathway in colon cancer. APC mutations result in the stabilization of β-catenin, leading to deregulation of β-catenin/TCF target gene expression and uncontrolled cell proliferation. The TCF4 knockout mouse model demonstrates the critical role of the β-catenin/TCF complex in regulating intestinal epithelial cell proliferation (16). Although these mice die shortly after birth, it was noted that they experience a complete failure of proliferation in the stem-cell compartment of their small intestine. More severe developmental defects were recently reported for the TCF4−/−/TCF1−/− double knockout mice (17). Together, these findings suggest the β-catenin/TCF complex plays a critical role in regulating intestinal epithelial cell development and proliferation.

Development and differentiation of the intestinal epithelium are also regulated in part by the activity of the homeodomain proteins Cdx1 and Cdx2 (18–24). Cdx1 and Cdx2 are transcription factors required for the intestine-specific expression of a...
number of genes (20, 23, 25). Cdx1 and Cdx2 also promote differentiation of intestinal cells (19, 21, 22). Observations from a number of laboratories suggest these factors can regulate epithelial cell proliferation. There is evidence that Cdx2 expression reduces intestinal epithelial cell proliferation and behaves like a tumor suppressor (21, 26, 27). However, the role of Cdx1 in the control of intestinal cell proliferation is less well understood.

Cdx1 is expressed in the intestinal crypts, and Wnt/β-catenin signaling is required for Cdx1 expression (28, 29), suggesting a role for Cdx1 in promoting proliferation. However, Cdx1 expression is preferentially silenced with the progression to colon cancer in humans (30, 31), casting doubt on the premise that Cdx1 promotes proliferation. In our previous studies (19, 32, 33), we used inducible or transient methods to express Cdx1, and we observed that Cdx1 expression inhibited cell proliferation. We determined this was due to Cdx1-mediated inhibition of G1 cyclin-dependent kinase activity. Cyclin D1 mRNA and protein levels were diminished by Cdx1 expression, and overexpression of cyclin D1 inhibited the G0/G1 block. Finally, cyclin D1 mRNA stability studies, time course analyses, and cyclin D1 promoter studies suggested that Cdx1 acts to diminish cyclin D1 gene transcription rather than utilizing post-transcriptional and post-translational mechanisms that have been described for cyclin D1 (34, 35).

Here we analyze in greater detail the molecular basis of Cdx1- and Cdx2-mediated inhibition of colon cancer cell proliferation. We noted that cyclin D1 has been implicated as a transcriptional target of β-catenin/TCF, and we hypothesized that Cdx1 expression reduced cyclin D1 gene expression by modulating β-catenin/TCF transcriptional activity. We establish that Cdx1 and Cdx2 expression does inhibit β-catenin/TCF transcriptional activity. This inhibitory effect is dose-dependent and can be observed in a number of different colon cancer cell lines. Moreover, the degree to which Cdx1 inhibits β-catenin/TCF transcriptional activity correlates with the ability of Cdx1 to reduce cell proliferation. Furthermore, we demonstrate that Cdx1 expression can inhibit β-catenin/TCF-mediated inductions of cyclin D1 and Cdx1 promoter activity. Cdx1 expression does not alter β-catenin protein levels or intracellular distribution nor does it induce an inhibitory TCF isoform. We also find that despite the observation that the Cdx1 gene is a Wnt/β-catenin/TCF transcriptional target, Cdx1 expression is lost in Min mouse polyps with increased nuclear localization of β-catenin. Finally, we show that colon cancer cells effectively reduce Cdx2-mediated inhibition of Wnt/β-catenin/TCF transcriptional activity as compared with other cells and model systems. This suggests that colon cancer and possibly crypt epithelial cells can modulate Cdx2’s effect upon β-catenin signaling and proliferation. We conclude that Cdx1 and Cdx2 inhibit colon cancer cell proliferation by blocking β-catenin/TCF transcriptional activity.

MATERIALS AND METHODS

Cell Culture, Adenoviral Infection, and Transfections—DLD1, HT-29, SW480, 293-T, and HCT116 cells were obtained from the ATCC or the Cell Center (University of Pennsylvania). The cells were all maintained as recommended by the ATCC. Adenovirus infections were performed as described (32, 33). The multiplicity of infection yielding >90% of cells expressing Cdx1 had been determined previously (32, 33). A multiplicity of infection of 67 was determined to be optimal for DLD1 cells (32) and was used in these experiments. At 48 h postadenoviral infection, the cells were processed for the various end points including protein and RNA isolation.

293-T cells were transfected using FuGENE 6 (Roche Applied Science). DLD1, HCT-116, and HT-29 cells were transfected by electroporation as described (32, 36). Cells at 70–80% confluence were suspended by treatment with trypsin, washed with PBS, and electroporated with 3 μg of pRC expression vectors, 1 μg of TOPFLASH or FOPFLASH reporter (kindly provided by Ken Kinkler, The Johns Hopkins University), 25 ng of pRL-CMV Renilla control reporter (Promega), and 31 μg of Bluescript II SK+ (Stratagene) as carrier. Media were changed at 24 h, and at 48 h the cells were harvested by trypsin treatment and washed once in PBS. Luciferase assays were carried out by using the dual luciferase assay kit. Luciferase activity was normalized to both total protein concentration and the transfection control Renilla luciferase levels. Cellular DNA content was quantified as described (32). Cells were transfected by electroporation using a Pulse System II (Bio-Rad). Media were changed at 24 h, and at 48 h the cells were harvested and fixed in PBS, 70% EtOH at −20 °C. The following day the cells were washed and stained with propidium iodide.

DNA content was quantified by flow cytometry, with gating on GFP+ cells.

A human cyclin D1 promoter-luciferase reporter construct (D1pro-1748 (PvuII)) was provided by Dr. Anil Rustgi (University of Pennsylvania) (37). Cdx1 truncation constructs were synthesized by PCR using oligonucleotide primers to amplify Cdx1 truncations from a murine cDNA template as described (32, 33). The human Cdx1 (−600)–luciferase reporter was described previously (38). The truncated cyclin D1 promoter (CD−960) and the CD−960 mutant promoter (TCF−(1−4)) were kindly provided by Ifor Tetu and Frank McCormick, University of California, San Francisco (15).

Immunoblot, and Immunohistochemical and Immunoprecipitation Analyses—Several 100-mm plates of DLD1 cells were infected and cultured. At 48 h, they were suspended by trypsin treatment and washed twice in PBS, and then whole-cell protein extracts were prepared as described previously (32, 33). The products analyzed by SIS-PAGE and immunoblotting. Cytoplasmic and nuclear fractions were prepared as described previously (39). The antibodies for Cdx1 (CPSP) and Cdx2 (CNP) have been described previously (30, 39). The β-catenin antibody was mouse monoclonal 610153 (Transduction Laboratories). For Western blot loading control, we used the actin:A-4700 (Sigma). For the β-catenin co-immunoprecipitation study, DLD1 cells were infected by adenovirus as before. At 48 h post-infection, cells were harvested and lysed in mammalian protein extract reagent (M-PER, Pierce). Immunoprecipitation was performed by using the anti-β-catenin antibody (sc-7199, Santa Cruz Biotechnology) according to the manufacturer’s instructions. The precipitated products were analyzed by Western blotting by using an anti-TCF4 monoclonal antibody (sc-8631, Santa Cruz Biotechnology) and anti-β-catenin (sc-7963, Santa Cruz Biotechnology).

Immunofluorescence and Immunohistochemistry—DLD1 cells were transfected and cultured as described. At 48 h cells were fixed with 4% paraformaldehyde, permeabilized, stained for Cdx1 (using polyclonal CDS) and β-catenin (mouse monoclonal antibody, Transduction Laboratories), and then followed by the secondary antibodies cy2-antimouse and cy3-anti-rabbit (Jackson Immunoresearch). The cells were examined by fluorescent microscopy, and images were obtained by using a Photometrix Coolsnap-CF Black and White CCD camera (Roeper Scientific) mounted on a Nikon E860 fluorescent microscope. Min mouse polyps were kindly provided by Greg Enders (University of Pennsylvania). Each sample was fixed in 10% neutral buffered formalin and paraffin-embedded for immunohistochemical staining. Sections were immersed in 0.1 mol/liter citrate buffer (pH 6.0) and microwaved for 10–13 min. The slides were deparaffinized and then treated with 30% hydrogen peroxide and avidin/biotin according to the protocol provided by Vector Laboratories. Samples were incubated overnight at 4 °C by using the following primary antibodies, Cdx1-CPSP (1:1000) and β-catenin (1:250, Transduction Laboratories). The slides were stained using 3,3′-diaminobenzidine tetrahydrochloride (Sigma), counterstained with hematoxylin, and mounted using Permount (Fisher).

Northern Blot and Ribonuclease Protection Analysis—Total RNA was isolated from adenovirus-infected DLD1 cells using the mirVana Kit (Ambion). The samples were incubated in 4 °C by using the following primary antibodies, Cdx1-CPSP (1:1000) and β-catenin (1:250, Transduction Laboratories). The slides were stained using 3,3′-diaminobenzidine tetrahydrochloride (Sigma), counterstained with hematoxylin, and mounted using Permount (Fisher).

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clones were treated with adeno-Cdx1 (white) and adeno-Null (gray) both at an multiplicity of infection of 67 or were left uninfected (black). At 48 h postinfection, luciferase activity was measured. n = 3 for each treatment. RLU, relative light units.

the previous probe and then prehybridized and hybridized as before.

Xenopus Wnt Reporter Assays—For the Xenopus Wnt reporter assays, 100 pg of LEF-fos plasmid (similar to TOPFLASH except that it contains seven TCF/LEF-binding sites upstream of a minimal promoter derived from the fos promoter (40) and 25 pg of pRL-SV40 (a Renilla luciferase control for injection)) were injected into the animal pole of fertilized eggs with mRNA encoding Xenopus wnt-1 and/or mouse cdx2 (100 pg of each mRNA/embryo). Embryos were harvested at the early gastrula stage (2 × 10 embryos per sample), and luciferase assay were performed with a dual luciferase assay kit (Promega), as described previously (41). Results for each group were normalized to Renilla luciferase activity. The mean from four independent experiments was plotted.

RESULTS

Cdx1 Expression Reduces c-Myc and Cyclin D1 Gene Expression in Human Colon Cancer Cells—In a previous study we established that expression of Cdx1 in human colon cancer cells inhibited proliferation by reducing cyclin D1 mRNA and protein levels (32). Initial transfection studies with a cyclin D1 promoter-reporter (∼1749)-Luc found Cdx1 reduced promoter activity by 20–30%. This reduction was modest compared with the 60–75% reduction we typically observed in cyclin D1 mRNA and protein (Fig. 1A) (32). As the protein and mRNA determinations were carried out with adenovirally mediated Cdx1 expression, it was possible the discrepancy was due to differences in techniques. We therefore generated stable D1(∼1749)-Luc-transfected DLD1 cells to examine the promotor response to adeno-Cdx1 treatment. 80 individual clones were screened, and 8 were found to express luciferase activity. We tested seven clones for their response to adeno-Cdx1 treatment. We found adeno-Cdx1 reduced luciferase activity between 10 and 35% when compared with controls in five of the seven clones (Fig. 1B, clones 65, 41, 70, 26, and 37). One clone had an indeterminate response (clone 19), and luciferase levels were mildly induced in one clone (clone 9). In summary, Cdx1 expression reliably reduced cyclin D1 (−1749) promoter activity by an average of 20–30% in DLD1 cells.

Cdx1 Expression in Colon Cancer Cells Inhibits β-Catenin/TCF Reporter Activity—We next began investigating known mechanisms regulating cyclin D1 gene expression in human colon cancer cells. In previous studies, we noted that c-Myc mRNA levels were reduced by Cdx1 expression (Fig. 1A) to 40% of adeno-Null-treated cells and only 20% of uninfected cells. A microarray analysis comparing adeno-Cdx1- and adeno-Null-treated DLD1 cells found Cdx1 reduced cyclin D1 and c-Myc as well as LEF1 mRNA levels (data not shown). All three were reduced 3–4-fold. We recognized all three genes as transcriptional targets of the Wnt/β-catenin/TCF signaling pathway (10, 12, 15, 42). Given the critical role played by β-catenin/TCF transcriptional activity in promoting proliferation of human colon cancers and cyclin D1 expression (1, 16, 43–45), we hypothesized that Cdx1 expression reduced DLD1 proliferation and cyclin D1 and c-Myc mRNA levels by inhibiting β-catenin/TCF transcriptional activity. To test this, we co-transfected DLD1 cells with Cdx1 and a canonical β-catenin/TCF luciferase reporter, TOPFLASH (44, 45). As a control, we used the FOPFLASH vector in which the β-catenin/TCF-responsive elements (TRE) were mutated and no longer bound TCF. TOPFLASH luciferase activity was quite high in DLD1 cells. Luciferase levels were more than 20-fold greater than the FOPFLASH control after normalization (Fig. 2A). Co-transfection of a Cdx1 expression vector (Cdx1-wt) reduced TOPFLASH luciferase activity by 80–90% compared with empty vector controls. In contrast, FOPFLASH luciferase activity was increased 2–3-fold with Cdx1 expression. To investigate the specificity of this effect, we co-transfected a transcriptionally inactive Cdx1 mutant, Cdx1-ND (Cdx1(141–183)), in which the N terminus was deleted. In a previous study (32) we had determined that Cdx1-ND was unable to induce a sucrase-isomaltase (∼183 to +54) luciferase reporter and could act in a dominant-negative fashion to inhibit Cdx-mediated gene expression. Titration experiments with the Cdx1-ND and Cdx1-wt expression vectors suggest the repression is mediated by direct competition between wild-type Cdx proteins and the transcriptionally nonfunctional Cdx1-ND mutant for the DNA-binding sites in the promoters of responsive genes (data not shown). In contrast to the effect of the wild-type Cdx1, co-transfection of the Cdx1-ND truncation mutant increased TOPFLASH activity nearly 3-fold.

![Figure 1. Cdx1 expression reduces cyclin D1 and c-Myc mRNA levels and cyclin D1 promoter activity. A, DLD1 cells were treated with an adenovirus containing a Cdx1 cDNA (X1) at a multiplicity of infection of 67. At 48 h total RNA was isolated and subjected to sequential Northern analyses for cyclin D1, c-Myc, and the loading control 36B4. As controls, RNA was isolated from uninfected cells (C), and cells were treated with a control adenovirus, adeno-Null (N). B, DLD1 cells were stably transfected with the cyclin D1 promoter-reporter D1-pro (∼1749)-Luc. Individual clones were treated with adeno-Cdx1 (white) and adeno-Null (gray) both at an multiplicity of infection of 67 or were left uninfected (black). At 48 h postinfection, luciferase activity was measured. n = 3 for each treatment. RLU, relative light units.](http://www.jbc.org/doi/fig/1)

![Figure 2. Cdx1 inhibits β-catenin/TCF transcriptional activity. A, 36867 mRNAs and proteins were measured in DLD1 cells treated with and without Cdx1 (Cdx1-wt). B, 36867 mRNAs and proteins were measured in DLD1 cells treated with and without Cdx1 (Cdx1-wt).](http://www.jbc.org/doi/fig/2)
Cdx1 Inhibits β-Catenin/TCF Transcriptional Activity

TOPFLASH Activity Is Inhibited by Cdx2 Expression—Cdx2 expression has been reported to reduce the proliferation of human colon cancer cells in culture (21, 46, 47) and to inhibit β-catenin-mediated induction of Cdx1 expression (48). However, the mechanism for this effect is unknown. We investigated if Cdx2 expression could reduce TOPFLASH luciferase activity in a manner similar to Cdx1. We found that co-transfection of a Cdx2 expression vector did indeed diminish TOPFLASH luciferase activity. In HT-29 and DLD1 cells, Cdx2 expression reduced luciferase activity by 30–40% when compared with control cells (Fig. 4). In HCT116 cells, Cdx2 reduced TOPFLASH luciferase activity by nearly 70%. In summary, Cdx2 expression in human colon cancer cell lines can reduce β-catenin/TCF transcriptional activity. However, this effect is generally less than or equal to that observed for Cdx1 in colon cancer cells.

β-Catenin/TCF-mediated Target Gene Expression Is Inhibited by Cdx1—TOPFLASH is a sensitive and specific reporter construct to measure β-catenin/TCF transcriptional activity. To confirm observations made with TOPFLASH, we tested for the ability of Cdx1 to inhibit the β-catenin/TCF induction of the promoters for the human cyclin D1 and CDX1 genes. Both are known β-catenin/TCF transcriptional targets (10, 15, 29). We carried out these studies in 293T cells because their base-line β-catenin/TCF transcriptional activity is lower than in colon cancer cells and they have been used in many published reports for similar studies (10, 12, 13, 48).

Co-transfection of the human cyclin D1 promoter (--1749)-Luc reporter with a β-catenin expression vector induced luciferase activity by nearly 6-fold (Fig. 5A). 80% of the activity was lost with co-expression of Cdx1. In the absence of β-catenin, Cdx1 expression had no significant effect on luciferase levels. A truncated cyclin D1 promoter (CD –960) was induced only 2.5-fold by β-catenin. Although there are no known β-catenin/TCF-responsive elements (TRE) located between –960 and –1749 of the cyclin D1 promoter, the 2.5-fold induction by β-catenin in 293T cells is in agreement with the 3-fold enhancement reported by Tetsu and McCormick (15). Cdx1 co-expression significantly reduced this induction to nearly the same level as that of CD –960 with Cdx1 expression alone (Fig. 5A). The CD –960 promoter in which the β-catenin-responsive TRE elements have been mutated (15), TCF (0)–(4), did not respond to β-catenin expression, and Cdx1 expression weakly induced luciferase activity.

The Cdx1 gene is known to be a Wnt/β-catenin/TCF target gene. CDX2 has been reported previously (48) to inhibit β-catenin-mediated induction of CDX1 gene expression, but the ability of Cdx1 expression to inhibit its own promoter activity was not tested. Co-transfection of the CDX1 promoter (--600)-Luc reporter with β-catenin enhanced luciferase expression in 293T cells by 2-fold over controls (Fig. 5B). The addition of Cdx1 expression completely abrogated this induction. In the absence of β-catenin, Cdx1 expression had no effect on the activity of its own promoter. In summary, Cdx1 expression reduced cyclin D1 and CDX1 promoter activity by specifically inhibiting β-catenin-dependent transcription.

Cdx1 Expression Does Not Alter β-Catenin Levels or Intracellular Distribution—We next utilized β-catenin Western immunoblotting and immunohistochemistry to investigate mechanisms responsible for the effect of Cdx1 on TOPFLASH activity. To determine whether Cdx1 expression reduces β-catenin protein levels, whole-cell protein extracts were isolated from DLD1 cells at 48 h post adenovirally mediated Cdx1 expression. β-Catenin immunoblotting was then performed. There was no significant change noted in β-catenin total protein levels induced by Cdx1 expression when compared with...
adeno-Null-infected or uninfected control cells (Fig. 6A). Extranuclear sequestration of β-catenin has been demonstrated to reduce β-catenin/TCF-mediated proliferation and transcriptional activity (49–53). To determine whether extranuclear sequestration of β-catenin was induced by Cdx1 expression, we obtained nuclear and cytoplasmic protein extracts from our adenovirus-treated DLD1 cells. β-Catenin levels in nuclear extracts from adeno-Cdx1-treated cells did not significantly differ from controls (Fig. 6B). In another experiment, DLD1 cells were transiently transfected with Cdx1 or the truncation mutant Cdx1-ND and were then co-stained for Cdx1 and β-catenin. Epifluorescent and confocal microscopy confirmed that extranuclear sequestration was not induced. β-Catenin staining localized to the nucleus and the cell-cell boarders in all cells (Fig. 6C and data not shown). There were no significant differences in β-catenin localization when comparing transfected or untransfected cells nor were there differences between cells transfected with the wild-type or mutant Cdx1 (Fig. 6D). In summary, Cdx1 expression does not alter β-catenin protein levels or intracellular distribution.

We considered other mechanisms by which Cdx1 might inhibit β-catenin-mediated expression from the TOPFLASH and cyclin D1 promoters. To exclude the possibility that Cdx1 directly bound these promoters to inhibit activation or induce an inhibitory LEF1 or TCF1 isoform (42, 54, 55), we used a chimeric TRE transcription activator, VP16-THMG. This construct contains the Xte3 DNA binding domain (amino acids 316–411) ligated in-frame to the VP-16 transcription activation domain and will activate promoters containing a TRE. This chimera robustly activates TOPFLASH reporter activity.

### Table I

| Genotype and phenotype of cell lines used experimentally |
|---------------------------------------------------------|
| **Stability** | **APC genotype** | **β-Catenin genotype** | **Endogenous CDX1** | **Endogenous CDX2** |
|---------------|------------------|-----------------------|---------------------|---------------------|
| SW480         | MSS (84)         | Wild-type (84, 85)    | Absent (76, 77)     | Present (77)        |
| HT29          | MSS (84)         | Wild-type (84, 85)    | Absent (76, 77)     | Weakly present (76, 77) |
| DLD1          | MSI (84)         | Wild-type (84, 85)    | Absent (32, 76, 77) | Present (32, 76, 77) |
| HCT116        | MSI (84)         | Mutant (84, 85)       | Absent (76, 77)     | Weakly present (76, 77) |

Fig. 3. Inhibition of β-catenin reporter activity by Cdx1 expression in HCT-116 HT-29, and SW480 cells. As in Fig. 2, the β-catenin/TCF reporter TOPFLASH or the control FOPFLASH were co-transfected into HCT116 HT-29 or SW480 cells along with expression vectors for Cdx1 (white bars), the mutant Cdx1-ND (gray bars), or the empty vector control (black bars). Luciferase activity was determined and normalized as before. One of three experiments is shown in each case. A, transfection studies with HT-29 cells. B, transfection studies with HCT-116 cells. C, studies with SW480 cells. D, HCT116 and SW480 cells transfected with expression vectors for Cdx1, the truncation mutant Cdx1-ND, or the empty vector as control. A GFP expression vector was co-transfected to mark transfected cells. At 48 h, the cells were isolated, stained with propidium iodide, and analyzed for GFP expression and DNA content by flow cytometry. The percentage of transfected cells in G0/G1 was determined by gating on GFP+ cells only. This was then normalized to the transfection control (%GFP+). Eight separate transfections were performed, and the results for each were averaged, and the results were expressed as fold change when compared with the control pRC/CMV-transfected cells.
in the absence of β-catenin/TCF (Fig. 6D); however, TOPFLASH activity was increased to even greater levels by β-catenin co-transfection (Fig. 6D). Co-transfection of Cdx1 with β-catenin reduced TOPFLASH luciferase levels by 95%. When Cdx1 was co-transfected with VP16-THMG, rather than suppress TOPFLASH, it induced it weakly. Co-transfection of a dominant-negative TCF4 expression vector further inhibited TOPFLASH by nearly 70% (Fig. 6D). This suggests Cdx1-mediated inhibition of β-catenin/TCF target gene expression is not due to induction of an inhibitory TCF isoform or direct inhibition at the TRE.

In a final study, we tested to see if Cdx1 expression disrupted the interaction between β-catenin and TCF4. β-Catenin was immunoprecipitated from aden-Cdx1-treated DLD1 cells and control cells, and the quantity of TCF4 that co-precipitated with β-catenin was determined by immunoblotting. Cdx1 expression had no effect on the amount of TCF4 that was co-precipitated with β-catenin (Fig. 6E). Interference from immunoglobulin bands precluded an assessment of Cdx1 in the immunoprecipitating complex (data not shown).

Cdx1 Expression Patterns in a Murine Model of Colon Tumorigenesis—Given our findings in human colon cancer cell lines, we next investigated an in vivo model of carcinogenesis to compare patterns of Cdx1 and β-catenin expression. C57BL/6J(B) APCMin/+ mice are heterozygous for a germ line mutation in the APC. After acquiring a second somatic mutation in the wild-type APC allele, Min mice develop numerous adenomatous intestinal polyps due to unregulated β-catenin levels, β-catenin nuclear localization, and target gene expression. If Cdx1 expression promoted proliferation and polyp formation, as do other β-catenin target genes (10–15, 56), we would expect Cdx1 expression to be increased in the Min mouse polyps. We obtained four colonic polyps from different C57BL/6J(B) APCMin/+ mice. Adjacent sections were immunohasayed for either β-catenin or Cdx1 and then counterstained. Rather than being increased, Cdx1 expression was lost in all four Min mouse polyps (Fig. 7). Nuclear Cdx1 was detected in the normal cells adjacent to the poly (Fig. 7, arrowhead, 40×) as well as the normal cells of the polyp stalk. No nuclear Cdx1 staining was observed in the dysplastic cells of the poly (Fig. 7, arrow, 200×). By contrast, β-catenin staining was significantly greater in these cells, and nuclear staining also evident (Fig. 7, arrow, 200×). In summary, despite being a Wnt/β-catenin target gene, Cdx1 expression was lost in Min mouse polyps with increased nuclear localization of β-catenin.

Murine Cdx1 or Cdx2 Inhibits Wnt/β-Catenin Signaling in Xenopus Embryos—We were interested to learn if effects of Cdx1 on β-catenin/TCF could be exhibited in normal, nontransformed, or immortalized cells. The developing Xenopus embryo provided an accessible system for this study. The Wnt/β-catenin/TCF signaling pathway is critical during embryonic development for both dorsal axis formation early in development and mesodermal patterning during later phases (57). Xcad2 had been shown to inhibit Xwnt-8/β-catenin secondary axis induction by blocking the function of the Wnt target gene siamois (58). We confirmed that Cdx1 or Cdx2 mRNA injection inhibited Xwnt-1 secondary axis formation, similar to what was reported for Xcad2 and Xcad3 (58–61), and that both Cdx1 and Cdx2 caused posteriorization when expressed in dorsal blasomeres. We also noted that to achieve the same level of secondary axis inhibition, we needed to inject 5–10-fold more Cdx1 mRNA than Cdx2 mRNA (data not shown). This finding contrasts with our observations in colon cancer cells, where Cdx2 is generally less effective than Cdx1 at inhibition of β-catenin/TCF signaling.

Xcad2 has been shown to function downstream of siamois (58); however, it is also possible that the Cdx genes block the activation of β-catenin/TCF-dependent transcription, as was observed in colorectal cancer cells. We therefore co-injected a Xenopus consensus reporter for Wnt/β-catenin signaling, Leffos-luciferase (40), along with mRNA for Cdx2 and Xwnt-1, and we assayed for luciferase activity in early gastrula stage embryos. Co-injection of Xwnt-1 mRNA increased luciferase activity by 7-fold over Leffos-luciferase alone (Fig. 8A). Cdx2 mRNA also enhanced luciferase activity but only by 2-fold (Fig. 8A and data not shown). Co-injection of Cdx2 mRNA with Xwnt-1 blocked nearly all of the Xwnt-1-mediated enhancement of luciferase expression. Luciferase activity was only slightly greater than that seen with the Cdx factors alone (Fig. 8A and data not shown). Co-injection of a dominant-active chimeric form of TCF, which readily enhanced Leffos-luciferase activity, could not be inhibited by Cdx (data not shown). This sug-

![Fig. 4. Expression of Cdx2 inhibits β-catenin reporter activity in DLD1 and HT-29 cells.](image-url)
Cdx1 Inhibits β-Catenin/TCF Transcriptional Activity

Cdx1 inhibits β-catenin/TCF activity and not intracellular levels or distribution. A, DLD1 cells were infected with an adenovirus to express Cdx1 (adeno-Cdx1), a control adenovirus with an empty cassette (adeno-Null), or were sham-infected as a control. At 48 h whole-cell extracts were prepared, and an immunoblot for β-catenin

Cdx2 Is a Potent Inhibitor of Wnt/β-Catenin Signaling in 293T Cells—Given the significant differences we observed with Cdx2 inhibition of Wnt/β-catenin signaling in colon cancer cells and Xenopus embryos, we were interested in determining whether this was a feature of other non-colon cancer cells. 293T cells are a human embryonic kidney epithelial cell line transformed by the expression of the SV40 virus T-antigen. Co-transfection of the TOPFLASH reporter with a β-catenin expression vector induced luciferase activity by about 95-fold (Fig. 8 B). Co-transfection of only 10 ng of Cdx1 repressed luciferase levels by nearly 60% and 100 ng repressed it by 85%. When Cdx2 was used, 10 ng inhibited 90% of the luciferase

Fig. 6. Cdx1 inhibits β-catenin/TCF activity and not intracellular levels or distribution. A, DLD1 cells were infected with an adenovirus to express Cdx1 (adeno-Cdx1), a control adenovirus with an empty cassette (adeno-Null), or were sham-infected as a control. At 48 h whole-cell extracts were prepared, and an immunoblot for β-catenin

β-catenin was immunoprecipitated (IP) from adeno-Cdx1-treated DLD1 cells and control (Con) cells. The immunoprecipitation products were then immunoblotted (Western blot, WB) for TCF4 and β-catenin as described.
activity, and 100 ng nearly completely blocked TOPFLASH luciferase expression. In summary, in 293-T cells, Cdx2 is a more potent inhibitor of Wnt/β-catenin transcriptional activity than Cdx1.

**DISCUSSION**

The Caudal-related homeodomain transcription factors Cdx1 and Cdx2 are well established, critical regulators of the intestinal cell phenotype. They promote development of the intestinal epithelium during embryogenesis, enhance expression of numerous intestine-specific genes, and induce the expression of mature, columnar cell morphology (18, 19, 21, 22). There is no question as to whether the Cdx transcription factors promote intestinal cell differentiation, only as to how this occurs. However, it is less clear how these factors regulate intestinal cell proliferation (18). Our studies add to this discussion by demonstrating that Cdx1 or Cdx2 expression can inhibit an important determinant of proliferation in the intestinal crypt and human colon cancer cells, β-catenin/TCF transcriptional activity.

**Cdx Expression Inhibits β-Catenin/TCF Transcriptional Activity**—In our previous studies (32, 33), focused nearly entirely on the ability of Cdx1 to inhibit colon cancer cell proliferation, we demonstrated that Cdx1-mediated inhibition of proliferation depended upon reductions of cyclin D1 gene expression. In these studies we also noted reductions in the mRNA levels of other known β-catenin/TCF transcriptional targets (c-Myc and LEF-1). Our studies here were designed to test for an inhibition of the Wnt/β-catenin/TCF signaling pathway by the Cdx factors. By using the TOPFLASH/FOPFLASH reporter system, in which three canonical TCF-responsive elements are cloned upstream of a minimal promoter, we established that Cdx1 or Cdx2 expression could significantly inhibit β-catenin/TCF transcriptional activity in several colon cancer cell lines with different genetic features and that Cdx1 is generally a more effective inhibitor than Cdx2 in colon cancer cells. We demonstrated for Cdx1 that this is a specific response as it is dose-dependent and requires a transcriptionally active Cdx protein, and an N-terminally truncated Cdx1 can elicit a dominant-negative enhancement of TOPFLASH activity in certain cell lines. Moreover, the ability of Cdx1 to inhibit proliferation correlates well with the ability of Cdx1 to inhibit TOPFLASH luciferase expression. TOPFLASH transcriptional activity was repressed the greatest by Cdx1 in DLD1 cells. DLD1 cells also responded most strongly to Cdx1 expression with a G0/G1 growth arrest (32). SW480 cells, on the other hand, responded weakly on both measures. HCT116 cells fell between these extremes. In other studies presented here, we extended this fundamental observation. We demonstrated that Cdx1 expression could inhibit the β-catenin/TCF-dependent activity from the promoters of Wnt/β-catenin target genes like cyclin D1 and the Cdx1 gene itself. This argues that Cdx1 may function to feed back...
and negatively affect β-catenin/TCF signaling in intestinal crypt cells rather than promote proliferation. The induction of target genes that negatively feed back on signaling function is a common regulatory mechanism. In addition, this would not be the first negative feedback loop described for the Wnt/β-
catenin pathway (62–64). It is, however, a new function for Cdx1 and Cdx2.

Although we have not yet identified the mechanism by which Cdx inhibits β-catenin/TCF transcriptional activity, we have eliminated several possibilities. Cdx1 expression does not alter β-catenin protein levels or its intracellular distribution in colon cancer cells, nor does Cdx1 expression lead to the induction of a dominant-negative TCF isoform (42, 54, 55) or the disruption of the β-catenin/TCF complex. In ongoing experiments, we continue to explore whether the mechanism requires direct interactions between Cdx and the β-catenin/TCF complex or by indirect means such as the induction of a repressor protein or inhibition of a required β-catenin/TCF co-factor (65, 66).

Our findings in Min<sup>APC<sup>−/−</sup></sup> mouse polyps further suggest Cdx1 acts to negatively feed back and suppress β-catenin/TCF signaling. As a Wnt/β-catenin target gene, Cdx1 levels would be expected to increase in Min<sup>APC<sup>−/−</sup></sup>-polyps. We observed the opposite; Cdx1 levels were significantly diminished in these polyps. This argues that Cdx1 expression is preferentially silenced in the progression to neoplasia in the colon. Finally, our studies with Cdx2 in these model systems suggest some differences between the Cdx factors with respect to their ability to inhibit Wnt/β-
catenin signaling. In colon cancer cells, Cdx2 was generally less effective than Cdx1 at inhibiting β-catenin/TCF transcriptional activity; at best it was as effective in one cell line, HCT116 cells (Fig. 4). However, in Xenopus embryos or 293-T cells, Cdx2 was significantly more effective than Cdx1. Cdx1 remained an effective inhibitor in all these model systems, and it was the relative ability of Cdx2 that appeared to change significantly. This suggests the ability of Cdx2 to inhibit β-catenin may itself be subject to complex regulatory mechanisms.

The Role of Cdx1 and Cdx2 in the Regulation of Cell Proliferation—The role of the Cdx homeodomain transcription factors in the regulation of cell proliferation is presently unclear.

Cdx1 has been described as an oncogene due to its reported ability to induce soft agar growth in 3T3 and IEC6 cells (22, 67), induce proliferating cell nuclear antigen expression and bromodeoxyuridine incorporation (68), inhibit p21 expression (69), as well as promote proliferation in IEC6 and CaCo2 cells (22, 46). In addition, Cdx1 mRNA levels are reportedly increased in some early stage adenomas based on semiquantitative reverse transcriptase-PCR analysis of polyp lysates (70). Similarly, Cdx2 expression in IEC-6 cells can increase cell proliferation by inducing growth factor gene expression (71, 72), and ectopic Cdx2 expression can be the transforming event in a mouse model for AML (73).

In contrast to the above reports, there are a number of other published studies (19, 32, 33) finding that Cdx1 expression reduced rather than increased the proliferation of cells in culture, and that a stable, inducible Cdx1 expression vector did not induce 3T3 cell soft agar growth. Additionally, Cdx1 expression is recognized as being diminished and actively silenced in human colon cancers (30, 31), a finding that is widely supported (70). Moreover, in that recent study of human polyps, more polyps had either no change or reduced Cdx1 mRNA (12 of 19) levels than were increased (7 of 19), despite frequent activation of the Wnt/β-catenin signaling pathway in human adenomatous polyps. Perhaps most importantly, Cdx1 protein levels did not appear to correlate with their PCR findings, based on the presented images, nor did they try to correlate them with β-catenin protein levels and intracellular distribution. As for Cdx2, studies in many cell lines have found it reduces proliferation (21, 46, 47, 74). Moreover, Cdx2 expression is lost in a subset of colon cancers, possibly by the action of a currently unidentified dominant-acting transcriptional repressor (75, 76). One finding that is difficult to explain is that our SW480 cells express considerable amounts of their endogenous Cdx2 (data not shown) which is in agreement with an earlier report (77). However, Hinoi et al. (76) reported little expression of endogenous Cdx2 in their SW480 cells. We have no explanation for this discrepancy except to raise the possibility the two SW480 cell lines are not the same cells despite their common name. Finally, two recent studies (27, 78) have provided compelling in vivo evidence that Cdx2 behaves as a tumor suppressor in intestinal epithelium. Together, these findings point to a complex role for the Caudal-related factors in regulating cell proliferation. It suggests that the response to Cdx expression may depend upon the cellular and tissue context to which the expression occurs. Untangling the complexities present in the response is an important area for future investigations.

A Model for Cdx1 and Cdx2 Inhibition of Proliferation in the Normal Intestinal Epithelium—Given the findings reported here, many additional questions emerge. Foremost among them is how do we reconcile our observations with events occurring in vivo in intestinal crypt cells? The intestinal and colonic epithelium is constantly renewed from proliferating cells residing in the crypt base. Proliferation in these cells is believed to depend upon β-catenin/TCF transcriptional activity (16, 44, 45, 79). Cdx1 and Cdx2 are both expressed in proliferating crypt cells, and their presence in these cells has been difficult to reconcile with a role for these factors as antiproliferative agents (18).

Colon cancer cells have been described as expressing phenotypes of crypt progenitor cells (79). Our observations in colon cancer cells suggest they are not equally sensitive to the inhibition of proliferation and β-catenin/TCF transcriptional activity by the Cdx factors. For example, Cdx1 expression in SW480 cells is not associated with strong reductions in β-catenin/TCF activity or proliferation. We therefore speculate that crypt cells may not be equally sensitive to the antiproliferative effect of Cdx. There may exist mechanisms within these cells to blunt the antiproliferative effects of Cdx1 and Cdx2. Moreover, cancer cells in general appear to diminish specifically the ability of Cdx2 to block proliferation. Although some colon cancers specifically silence CDX2 expression (75, 76), the majority does
not. Therefore, colon cancers that do not silence CDX2 expression may utilize additional mechanisms that limit CDX2-mediated inhibition of β-catenin/TCF transcriptional activity, thus effectively reducing the antiproliferative effects of CDX2.

Together, these observations suggest a model for CDX and β-catenin/TCF interactions in intestinal crypt cells (Fig. 9). β-Catenin/TCF promote the expression of target genes that enhance cell proliferation like cyclin D1 and c-Myc. CDX1 expression is also enhanced by β-catenin/TCF activity, and CDX2 may enhance CDX2 expression via an autoactivation pathway described previously for CDX2 (80). CDX1 and CDX2 act to slow cell proliferation and differentiation (83).

Future studies will be directed at testing this model and the hypotheses upon which it is based.

In summary, our present study establishes that CDX1 and CDX2 expression can inhibit β-catenin/TCF transcriptional activity. The degree to which CDX1 inhibits β-catenin/TCF transcriptional activity correlates with the ability of CDX1 to reduce cell proliferation. This is likely to be an important mechanism by which the CDX factors regulate the proliferation of intestinal crypt epithelial cells and helps to explain why CDX1 and CDX2 expression is lost or functionally inhibited during carcinogenesis in the colon. Moreover, our findings suggest a model by which CDX2 expression coordinates cell cycle withdrawal with the induction of differentiation. Our observations also suggest that colon cancer and possibly crypt epithelial cells can modulate the effect of CDX2 on β-catenin signaling and proliferation. We conclude that CDX1 and CDX2 inhibit colon cancer cell proliferation by blocking β-catenin/TCF transcriptional activity.

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Rong-Jun Guo, Edward Huang, Toshihiko Ezaki, Neesha Patel, Kristen Sinclair, Jinling Wu, Peter Klein, Eun-Ran Suh and John P. Lynch

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