Cell, Vol. 111, 241–250, October 18, 2002, Copyright ©2002 by Cell Press

The β-Catenin/TCF-4 Complex Imposes a Crypt Progenitor Phenotype on Colorectal Cancer Cells

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Summary

The transactivation of TCF target genes induced by Wnt pathway mutations constitutes the primary transforming event in colorectal cancer (CRC). We show that disruption of β-catenin/TCF-4 activity in CRC cells induces a rapid G1 arrest and blocks a genetic program that is physiologically active in the proliferative compartment of colon crypts. Coincidently, an intestinal differentiation program is induced. The TCF-4 target gene c-*MYC* plays a central role in this switch by direct repression of the *p21*$^{CIP1/WAF1}$ promoter. Following disruption of β-catenin/TCF-4 activity, the decreased expression of c-MYC releases *p21*$^{CIP1/WAF1}$ transcription, which in turn mediates G1 arrest and differentiation. Thus, the β-catenin/TCF-4 complex constitutes the master switch that controls proliferation versus differentiation in healthy and malignant intestinal epithelial cells.

Introduction

The colorectal mucosa contains large numbers of invaginations termed the crypts of Lieberkühn. Epithelial renewal occurs in these crypts through a coordinated series of events involving proliferation, differentiation, and migration toward the intestinal lumen. Pluripotent stem cells at the crypt bottom generate progenitors that occupy the lower third of the crypt. Cells in this amplification compartment divide approximately every 12 hr. In the midcrypt region, the cells differentiate into one of the functional cell types of the colon. At the epithelial surface, cells undergo apoptosis and/or extrusion into the lumen. The entire process takes approximately 3–5 days (Potten and Loeffler, 1990).

The transition of an intestinal epithelial cell into a fully transformed, metastatic cancer cell requires mutations in multiple proto-oncogenes and tumor suppressor genes (Kinzler and Vogelstein, 1996). The *APC* gene, originally cloned from the rare genetic disorder familial adenomatous polyposis, is mutated in most sporadic CRCs. The APC protein resides in the destruction complex, together with GSK3β, axin/conductin, and β-catenin. In this complex, phosphorylation by GSK3β targets β-catenin for ubiquitination and destruction by the proteasome. Wnt signaling inhibits GSK3β activity. Then, β-catenin accumulates in the nucleus, where it binds members of the TCF family and converts these WNT effectors from transcriptional repressors into activators (reviewed in Bienz and Clevers, 2000). In cancer, truncating mutations in *APC* and axin/conductin, as well as mutations in the GSK3β-target residues in β-catenin, all lead to the formation of constitutive nuclear β-catenin/TCF complexes (Korinek et al., 1997; Morin et al., 1997; Rubinfeld et al., 1997; Satoh et al., 2000; Liu et al., 2000). Activating mutations of the WNT pathway are the only known genetic alterations in early premalignant lesions in the intestine, such as aberrant crypt foci and small polyps (Powell et al., 1992). Thus, these mutations may initiate the transformation of colorectal epithelial cells.

In the intestinal epithelium, Tcf-4 is the most prominently expressed TCF family member (Korinek et al., 1997). Gene disruption has revealed that Tcf-4 is required to establish the proliferative progenitors of the prospective crypts in the embryonic small intestine (Korinek et al., 1998). A second TCF family member, TCF-1, is expressed in the intestinal epithelium predominantly as a dominant-negative isoform, which lacks the N-terminal β-catenin interaction domain. Genetic evidence suggests that Tcf-1 acts as an antagonist of Tcf-4 in the formation of polyps in an *APC*$^{+/-}$ background (Roose et al., 1999).

To understand the contribution of constitutive β-catenin/TCF-4 activity to the colorectal transformation process, we have undertaken a large-scale analysis of the downstream genetic program activated by β-catenin/TCF in CRC cells. We have subsequently analyzed the expression and activities of individual β-catenin/TCF-4-regulated genes in a physiological context. Our results indicate that the β-catenin/TCF-4 complex, through its control over c-MYC and p21$^{CIP1/WAF1}$ activity, inhibits differentiation and imposes a crypt progenitor-like phenotype on CRC cells.

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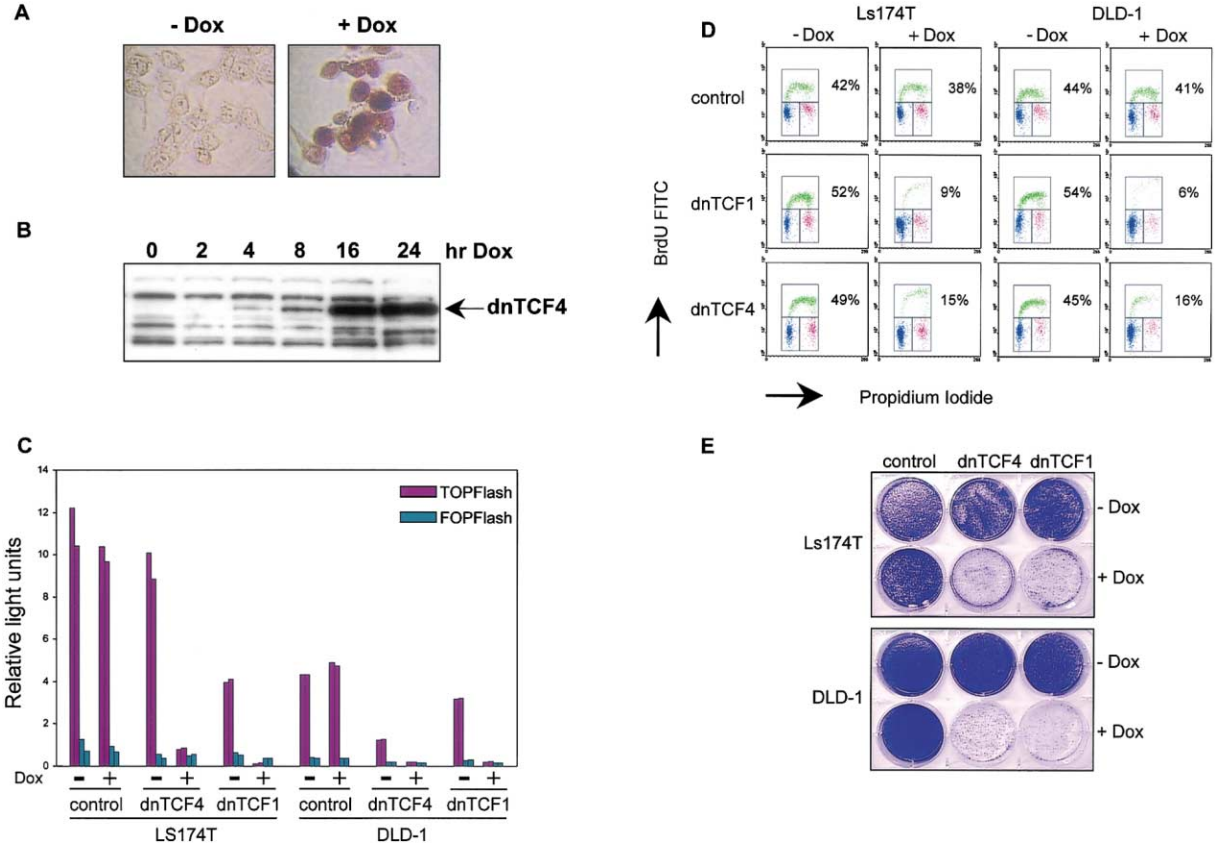

Figure 1. Inhibition of TCF/β-Catenin-Driven Transactivation by Induction of dnTCFs Results in Cell Cycle Arrest

(A) dnTCF-4 is highly expressed in the nucleus of induced cells. Ls174T cells stained for dnTCF-4 expression 24 hr after induction with doxycycline.

(B) dnTCF-4 protein is induced as early as 4 hr after induction with doxycycline as analyzed by Western blot.

(C) Both dnTCF1 and dnTCF-4 abrogate β-catenin/TCF driven transcription in the β-catenin mutant Ls174T as well as in the APC mutant DLD-1 cells. Activity of the TCF-reporter TOPFlash (purple bars) and control FOPFlash (green bars) after 24 hr with or without doxycycline (dox) is shown. Parental cell lines expressing the Tet-repressor alone are used as controls. Renilla luciferase levels were used as transfection controls.

(D) Ls174T and DLD-1 show a dramatic reduction in S phase cells upon dnTCF expression. The scatter profile of cells in G1 (blue), S (green), and G2/M (red) after 20 hr with or without doxycycline is shown. Numbers refer to the percentage of cells in S phase for each cell line analyzed. The results are representative of several independent experiments.

(E) Proliferation was halted in Ls174T and DLD-1 transfectants. This was visualized by crystal violet staining of cell cultures after 5 days of dnTCF expression.

Results

Inhibition of β-Catenin/TCF in CRC Cells Results in Cell Cycle Arrest

To determine the role of β-catenin/TCF complexes in CRC, we constructed cell lines carrying doxycycline-inducible expression plasmids encoding N-terminally truncated versions of TCF factors. Such dominant-negative TCF (dnTCF) proteins do not bind β-catenin and act as potent inhibitors of the endogenous β-catenin/TCF complexes present in CRC. Similar dnTCF factors have previously been instrumental to us in placing TCF factors within WNT pathways in frogs, flies, and worms (Molenaar et al., 1996; van de Wetering et al., 1997; Korswagen et al., 2000). As the recipient CRC cell lines, we chose Ls174T, which expresses mutant β-catenin protein, yet is diploid and carries wild-type alleles of *p53* and *APC*, and DLD-1, which is predominantly diploid and mutant for *p53* and *APC*. Strong nuclear dnTCF-4 staining was observed after 4–8 hr of doxycycline (Dox) induction (Figures 1A and 1B). CRC cell lines that carry WNT pathway mutations constitutively activate TCF reporters (pTOPFlash) (Korinek et al., 1997). We selected several clones in which the expression of dnTCF-4 completely abrogated this constitutive pTOPFlash activity (Figure 1C).

Induction of dnTCF-4 or dnTCF-1 in both cell lines resulted in a robust G1 arrest (Figure 1D). Accordingly, cell proliferation was halted as visualized by crystal violet staining (Figure 1E). No apoptosis was noted (not shown). We also generated cell lines expressing the β-catenin binding N terminus of TCF-4 fused to a nuclear localization signal (NLS) (N-TCF-4, see Supplemental Figure S1A at http://www.cell.com/cgi/content/full/111/2/241/DC1). The induction of N-TCF-4 abrogated β-catenin/TCF activity and had comparable effects on the cell cycle and gene profile of Ls174T cells as dnTCF (Supplemental Figures S1B–S1E).

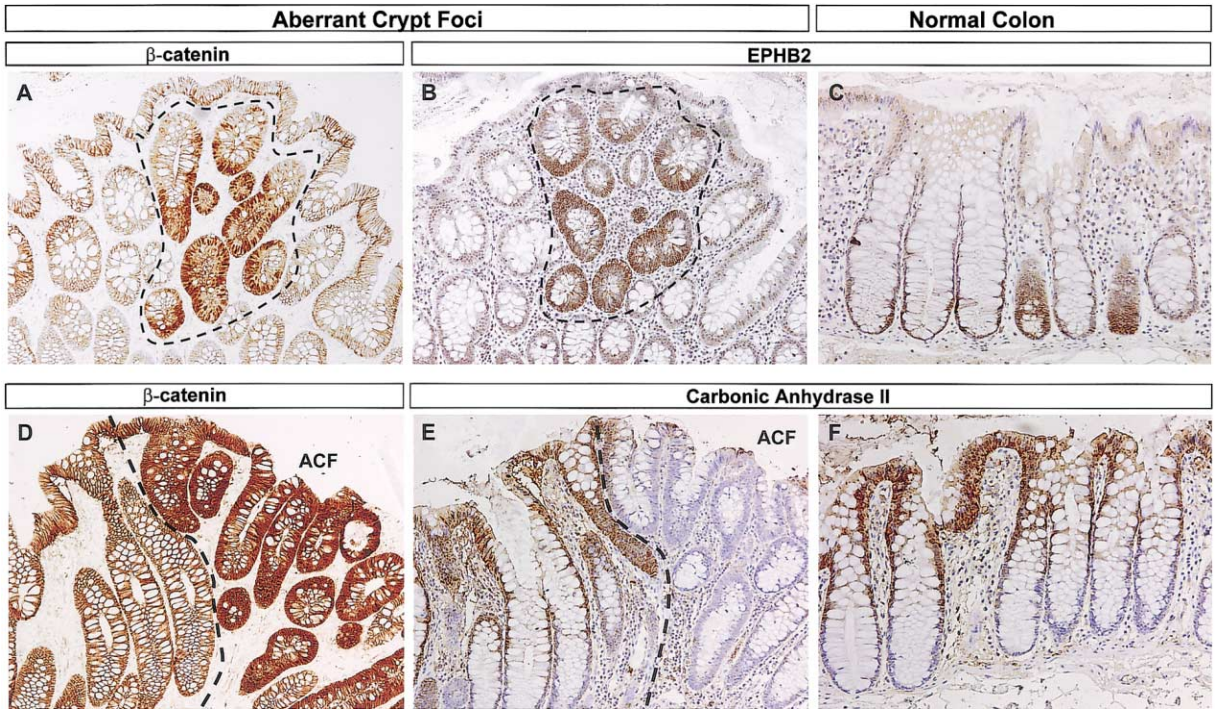

Figure 2. Genes Downstream of β-Catenin/TCF Activity Show a Complementary Expression Pattern to Intestinal Markers of Differentiation

Representative examples of the inverse expression domain of downregulated (B and C) or upregulated (E and F) genes in our system, in normal colon (C and F) or early colorectal lesions (B and E).

(A and B) The expression of nuclear β-catenin (A) perfectly correlates with that of EPHB2 tyrosine kinase receptor (B) in aberrant crypt foci (ACF). Stainings were performed on serial sections of early human lesions. The dashed lines delimit the same ACF in both stainings.

(C and F) EPHB2 and CAII show a complementary domain of expression in healthy tissue. EPHB2 is expressed at the bottom of the crypts (C), while CAII is only present in cells at the top of the crypts and surface epithelium of the colon (F).

(D and E) ACFs that colonize the surface epithelium show strong cytoplasmic and nuclear β-catenin (D) and are completely negative for carbonic anhydrase II (CAII) (E). The dashed line indicates the boundary between normal tissue and the ACF areas in serial sections.

The Genetic Program Driven by β-Catenin/TCF in CRC Cells

Previous studies have identified individual target genes of TCFs in various cellular systems (reviewed in www.stanford.edu/~rnusse/wntwindow.html). By DNA array analysis, we asked which genes were regulated by the induction of dnTCF-4 in Ls174T CRC cells. mRNA was isolated at 11 hr and 23 hr of induction. cDNA prepared from uninduced samples was labeled with Cy3, while the induced cDNA samples were labeled with Cy5. Uninduced and induced cDNA samples were mixed and hybridized in duplicate to a DNA array of approximately 24,000 cDNA entries. Fluorescent images were analyzed as detailed in Experimental Procedures. The complete data sets are available as Supplemental Figure S2 at http://www.cell.com/cgi/content/full/111/2/241/DC1.

We applied a single criterion to the array data set, a decrease of at least 2-fold in both measurements at 23 hr. This defined a small set of 120 downregulated entries (see Supplemental Table S1). Five genes (i.e., *CD44, BMP4, claudin-1*, *ENC1*, and c-*MYC*) had been described previously as TCF targets in CRC (Wielenga et al., 1999; Miwa et al., 2001; Kim et al., 2002; Fujita et al., 2001; He et al., 1998). *CD44*, c-*ETS2,* and nine other entries occurred twice, indicative of the robustness of the analysis (Supplemental Table S1). For a number of genes, we performed Northern blot analysis. Almost invariably, the genes listed in Supplemental Table S1 were also strongly downregulated by dnTCF-1 in Ls174T (Supplemental Figure S3). DLD-1 cells showed a highly similar pattern of gene expression upon inhibition of β-catenin/TCF activity (Supplemental Figure S3).

The expression of 115 entries increased at least 2.5-fold after 23 hr induction of dnTCF-4 in Ls174T cells (Supplemental Figure S2). Ten entries appeared twice in this list. Many of these genes represented differentiation markers of mucosecretory and/or absorptive intestinal cells (Supplemental Table S2). The induction of several RNAs was confirmed by Northern blotting (Figure 6F and Supplemental Figures S1 and S3E). We concluded that the inhibition of β-catenin/TCF-4 activity induced cell cycle arrest and differentiation.

The Genetic Program Controlled by β-Catenin/TCF in CRC Cells Is Physiologically Active in Colonic Epithelium

Immunohistochemical analyses were then performed on early neoplastic lesions. A strict correlation between nuclear β-catenin (Figure 2A) and the expression of target genes such as EPHB2 (Figure 2B) was observed in early colorectal lesions. Many other downregulated genes listed were overexpressed in early intestinal polyps from *Min* mice or in aberrant crypt foci (ACF) from

FAP patients (Figure 7, Supplemental Figure S4; Ramsay et al., 1992; Wielenga et al., 1999; Ciclitira et al., 1987; Erisman et al., 1985; Oba et al., 2001; Kitahara et al., 2001; Miwa et al., 2001). More strikingly, all tested target genes were expressed also in the proliferative compartment of normal colon crypts (*EPHB2*, Figure 2C; c-*MYB, BMP4, ENC1*, Figure S4; *CD44*, Figure 7; *EPHB3*, not shown). Several other downregulated genes from our list (*claudin-1*, c-*MYC*, and the Na-K-Cl cotransporter *SLC12A2*) are reportedly expressed in crypt cells (Miwa et al., 2001; Melhem et al., 1992; Matthews et al., 1998).

A complementary domain of expression was observed for genes strongly upregulated upon induction of dnTCF-4 in Ls174T cells. One such example is carbonic anhydrase II, which was restricted to the top of the crypts and the surface epithelium (Figure 2F) but was absent from polyp cells arising in this area (Figure 2E).

Cells in the intervillus pockets of the developing intestine, the prospective crypts, express Tcf-4 and exhibit nuclear β-catenin (van Noort et al., 2002). Accordingly, several genes listed in Supplemental Table S1 were strongly expressed in the proliferative pockets of Tcf-$4^{+/-}$ embryos (Figures 3A–3C). In Tcf-$4^{-/-}$ littermates, the expression of β-catenin/TCF regulated genes was completely absent (Figures 3F–3H). The cells in the intervillus pockets of Tcf-$4^{-/-}$ animals adopted a differentiated phenotype as indicated by $p21^{CIP1/WAF1}$ and Fabp-l staining (Figures 3I and 3J). Thus, the changes in gene expression induced by inhibition of β-catenin/TCF activity in CRC cells recapitulated the physiological differentiation of crypt progenitor cells.

These findings implied the WNT cascade to be active in normal adult colonic epithelium. Further support for this notion is presented in Figure 4. As an adherens junction component, β-catenin decorates the basolateral membrane of epithelial cells. In addition to this, we detected β-catenin in the nuclei of cells within the bottom third of the crypts in a gradient, with highest levels at the bottom (Figures 4A–4C).

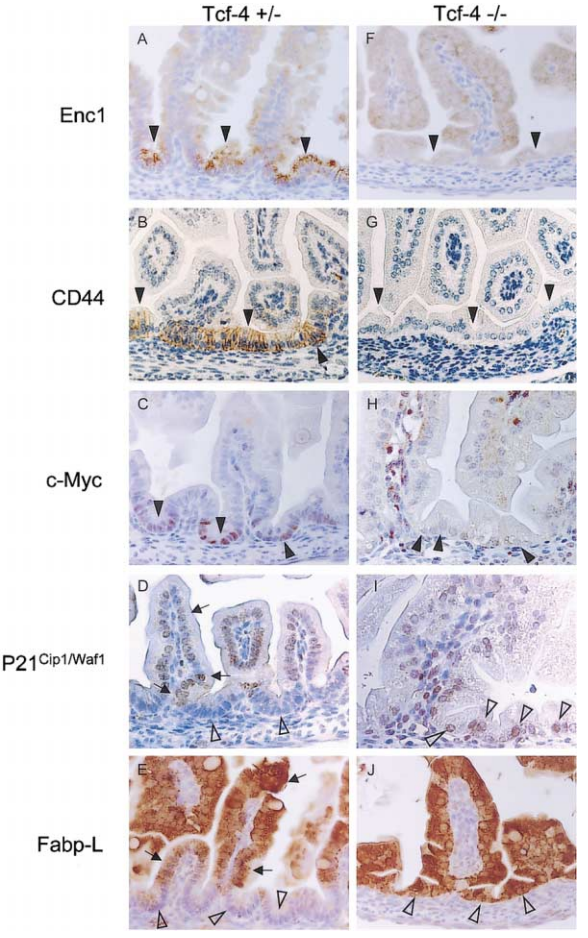

Figure 3. Genes Downstream of β-Catenin/TCF Activity Are Absent from the Prospective Crypts of Tcf-4 Null Mice

Immunohistochemical analysis of intestinal sections of Tcf$^{+/-}$ (A–E) and Tcf-$4^{-/-}$ mice (F–J).

(A–C, F–H) Genes downstream of β-catenin/TCF activity are expressed in the prospective crypts of Tcf-$4^{+/-}$ mice (A–C) but are absent in the same cells from Tcf-$4^{-/-}$ mice (F–H) (black arrowheads).

(D and E) Genes upregulated upon β-catenin/TCF inhibition in CRC cells are expressed in the differentiated cells of the villus of Tcf-$4^{+/-}$ mice (black arrows). Proliferative pockets are devoid of these stainings (white arrowheads).

(I and J) The prospective crypts from the Tcf-$4^{-/-}$ mice are occupied by cells expressing proteins characteristic of differentiated cells (white arrowheads).

$p21^{CIP1/WAF1}$ Mediates Cell Cycle Arrest and Differentiation

Which cell cycle regulators were regulated by induction of dnTCF in the DNA array experiment? *Cyclin D1*, a previously published TCF-4 target gene (Tetsu and McCormick, 1999; Shtutman et al., 1999), was not affected by the expression of dnTCF (Figure 5A). The only significant change in expression was observed for the CDK inhibitor *$p21^{CIP1/WAF1}$* (9-fold induction, Supplemental Table S2). This was readily confirmed by Northern (Figure 5A) and Western (Figure 5B) blotting. In accordance, the activity of the $p21^{CIP1/WAF1}$ substrate CDK2 was inhibited (Harper et al., 1993), as assayed by Histone H1 phosphorylation (Figure 5B). The *$p21^{CIP1/WAF1}$* induction was p53 independent, since the *p53* mutant cell line DLD-1 arrested in G1 and also upregulated *$p21^{CIP1/WAF1}$* (Figures 1D and 5A). Interestingly, *$p21^{CIP1/WAF1}$* marks the differentiated compartment of the colorectal epithelium (el Deiry et al., 1995).

We downmodulated *$p21^{CIP1/WAF1}$* levels by stable expression of antisense *$p21^{CIP1/WAF1}$* cDNA sequence. A significant reduction in the dnTCF-4-induced levels of *$p21^{CIP1/WAF1}$* was obtained (Figure 5C). In these cells, the cell cycle arrest induced by dnTCF-4 was greatly reduced (Figure 5D, p21AS).

Enforced cell cycle arrest is often sufficient to induce differentiation (Zhu and Skoultchi, 2001). The CIP/KIP family of CDK inhibitors comprises *$p21^{CIP1/WAF1}$*, $p27^{KIP1}$, and $p57^{KIP2}$ (Sherr and Roberts, 1999). The cell cycle arrest in our cells correlated with a strong induction of *$p21^{CIP1/WAF1}$*, while $p57^{KIP2}$ was not expressed and the levels of $p27^{KIP1}$ did not change. Inhibitors of the INK family were either not expressed ($p15^{INK4b}$, $p19^{INK4d}$) or were unaffected by dnTCFs ($p16^{INK4a}$) (not shown). To analyze the effects of $p21^{CIP1/WAF1}$ on differentiation, we generated Ls174T CRC cells inducibly expressing *$p21^{CIP1/WAF1}$* at levels comparable to those seen in dnTCF-expressing

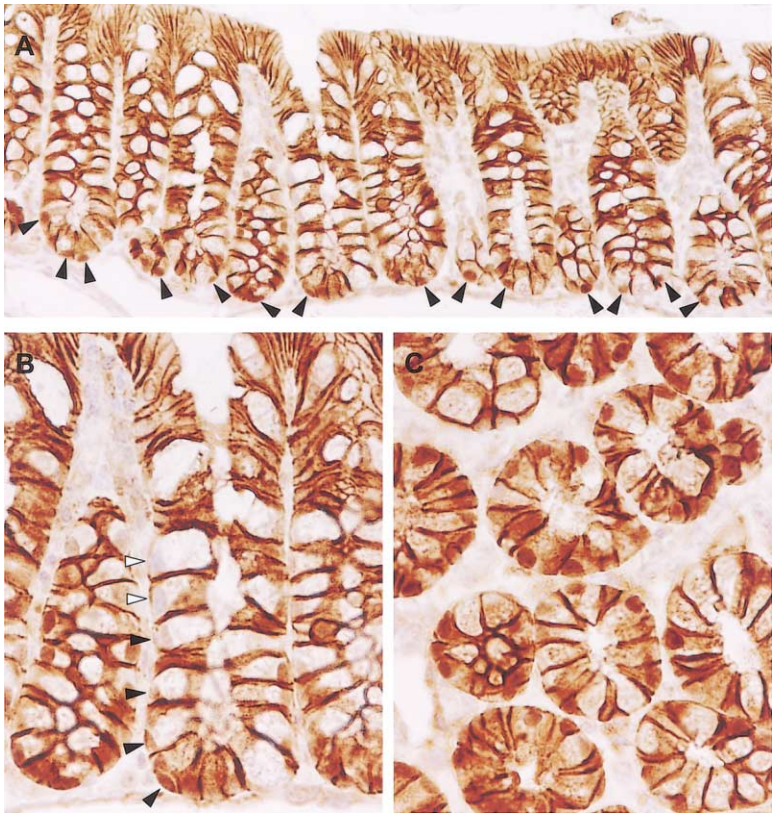

Figure 4. Nuclear β-Catenin Accumulates in the Proliferative Compartment of the Intestinal Crypts

β-catenin stainings in normal murine colon epithelium.

(A) Strong nuclear β-catenin can be observed in many cells at basal positions of colon crypts.

(B) Nuclear β-catenin staining decreases in a gradient as cells occupy higher positions toward the surface epithelium (black arrowheads). Nuclei from cells at the midcrypt region are negative (white arrowheads).

(C) Transverse section at the base of colon crypts showing many nuclei positive for β-catenin staining.

cells (Figure 5F). Induction of $p21^{CIP1/WAF1}$ resulted in a G1 arrest (Figure 5E) and in differentiation, as visualized by the upregulation of MUC-2, ephrin-B1, and galectin4 (Figure 5F).

c-MYC Mediates the Proliferative Effects of β-Catenin/TCF in CRC

BMP4, c-*MYC,* c-*MYB,* c-*ETS2*, and *ENC1* were tested for their ability to override the proliferative block induced by dnTCF by stable expression in Ls174T/dnTCF-4 cells. Clones that expressed the transfected gene at endogenous levels were selected for further studies. TOPFlash reporter assays indicated that none of the genes modulated the β-catenin/TCF pathway (not shown). By this add-back approach, we established that only c-MYC overrode the effect of dnTCF-4 on cell growth (Figure 6A) and $p21^{CIP1/WAF1}$ induction (Figure 6B, top), suggesting that c-MYC represses $p21^{CIP1/WAF1}$ expression. Indeed, we found that downmodulation of c-MYC by siRNA in Ls174T and DLD-1 CRC cells led to the upregulation of $p21^{CIP1/WAF1}$ (Figure 6C).

A 2.3 kb fragment of the $p21^{CIP1/WAF1}$ promoter sequence was cloned into a luciferase reporter vector and transiently transfected in Ls174T/dnTCF-4 cells. Upon induction of dnTCF-4, a 6- to 8-fold increase in activity of the promoter was observed (Figure 6D). Progressive deletions (not shown) mapped the responsive region to a 200 bp fragment in the proximal $p21^{CIP1/WAF1}$ promoter. The activity of this minimal promoter fragment followed that of the endogenous $p21^{CIP1/WAF1}$ gene, i.e., it was not activated upon induction of dnTCF-4 in the clones constitutively expressing c-MYC (Figure 6D).

The transcriptional activator MIZ-1 binds to the initiator of the $p15^{INK4b}$ promoter. c-MYC directly represses $p15^{INK4b}$ expression by binding to MIZ-1 (Staller et al., 2001; Seoane et al., 2001). We explored whether a similar mechanism could regulate $p21^{CIP1/WAF1}$. A similar mechanism is operative in the regulation of $p21^{CIP1/WAF1}$ in the UV damage response (Herold et al., 2002). Chromatin immunoprecipitation assays (ChIP) demonstrated that c-MYC and MIZ-1 are bound to the minimal $p21^{CIP1/WAF1}$ promoter (Figure 6E). Moreover, induction of dnTCF-4 greatly diminished the amounts of bound c-MYC. These results imply that c-MYC represses the cell cycle inhibitor $p21^{CIP1/WAF1}$ by direct promoter binding. The presence of MIZ-1 on this promoter fragment (Figure 6E) suggested a mechanism comparable to that reported for the control of $p15^{INK4b}$ by c-MYC.

Discussion

The Target Gene Program of β-Catenin/TCF in CRC Cells

The data presented here provide a global view of the genetic program driven by β-catenin/TCF activity in CRC cells. The expression of a surprisingly limited set of genes is dependent on the presence of active β-catenin/TCF complexes. In recent years, multiple TCF target genes have been described (reviewed in www.stanford.edu/~rnusse/wntwindow.html). Some of these (*CD44, ENC1, BMP4, claudin 1*) were also found in our study, while the expression of others was unaffected despite the complete suppression of TCF reporter gene activity in our system. Importantly, the downstream genes re-

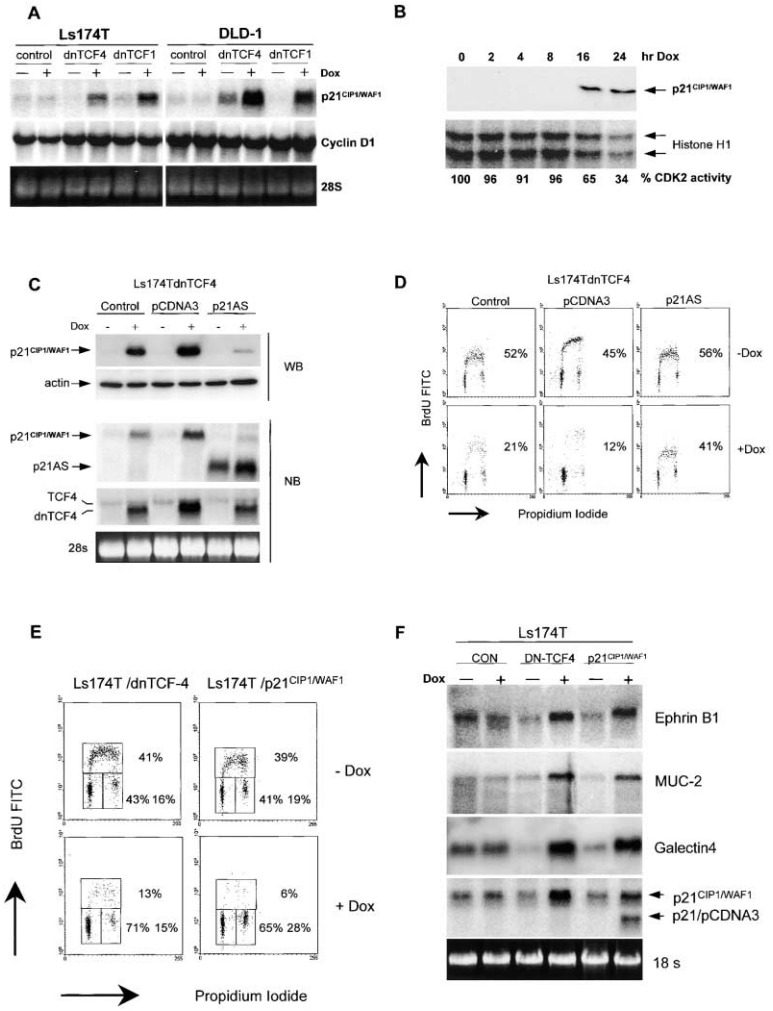

Figure 5. p21$^{CIP1/WAF1}$ Is an Important Mediator of Cell Cycle Arrest and Differentiation in Ls174T CRC Cells

(A) p21$^{CIP1/WAF1}$ mRNA was strongly upregulated upon inhibition of β-catenin/TCF activity (top). In contrast, *cyclin D1* mRNA does not change upon induction of dnTCFs (middle). The bottom gels show the 28S ribosomal RNA as a loading control.

(B) p21$^{CIP1/WAF1}$ protein was upregulated 16 hr after induction of dnTCF-4 with doxycycline in LS174T cells (top). The increase in p21$^{CIP1/WAF1}$ protein correlated with the inhibition of the cyclin-dependent kinase CDK2. Histone H1 phosphorylation is shown as a measurement of CDK2 activity (bottom).

(C) Downmodulation of p21$^{CIP1/WAF1}$ expression levels by antisense p21$^{CIP1/WAF1}$. Top: p21$^{CIP1/WAF1}$ protein levels before and after induction of dnTCF-4 in Ls174T/dnTCF-4 (control), Ls174T/dnTCF-4 transfected with empty pCDNA3 (pCDNA3), and Ls174T/dnTCF-4 transfected with full-length p21$^{CIP1/WAF1}$ cDNA in the antisense orientation (p21AS). Actin protein levels are used as a loading control. Bottom: Northern blot analysis of p21$^{CIP1/WAF1}$ and dnTCF-4 in the above cell lines.

(D) Downmodulation of p21$^{CIP1/WAF1}$ expression levels impairs the cell cycle arrest induced by dnTCF-4 expression. Scatter profile of cells after 24 hr with or without doxycycline. Results are representative of several independent experiments.

(E) Induction of p21$^{CIP1/WAF1}$ or dnTCF-4 causes a comparable G1 arrest in Ls174T cells. The cell cycle profile and the percentage of cells in each phase are shown after 24 hr treatment with or without doxycycline. Results are representative of several independent experiments.

(F) The expression of p21$^{CIP1/WAF1}$ in Ls174T CRC cells induces the upregulation of several differentiation markers.

ported here likely represent a transcriptional hierarchy. Some will be directly regulated by TCF-4 and will in turn control other genes in this hierarchy. The list of target genes facilitates our understanding of the first stages of colorectal tumorigenesis, but also of the physiology of the intestine. An example of the latter is the role of the EphB2 and EphB3 receptors in cell positioning in the intestine (Batlle et al., 2002 [this issue of *Cell*]).

Only one of the tested target genes rescued the cell cycle arrest imposed by dnTCF, i.e., c-*MYC*. c-MYC plays a central role in the proliferative capacity of many cancers, including CRC (reviewed in Grandori et al., 2000). Our data imply that c-MYC blocks the expression of the cell cycle inhibitor p21$^{CIP1/WAF1}$. We have mapped the region responsible for *p21$^{CIP1/WAF1}$* regulation to a 200 bp fragment of the proximal promoter. The presence of MIZ-1 and c-MYC on this promoter suggests that c-MYC-mediated repression of *p21$^{CIP1/WAF1}$* occurs by a mechanism resembling c-MYC control of p15INK4b, i.e., through preventing promoter activation by the transcription factor MIZ-1 (Staller et al., 2001; Seoane et al., 2001). Decreased expression of c-MYC would allow MIZ-1 to activate *p21$^{CIP1/WAF1}$* transcription (see model in Figure 6F). The complementarity in the expression of c-MYC and p21$^{CIP1/WAF1}$ in the intestine (Figures 3C and 3D; Melhem et al., 1992; el Deiry et al., 1995) supports this mechanism.

Ls174T cells that arrest in G1 upon expression of dnTCF recapitulate the physiological differentiation program of normal intestinal cells. Expression of p21$^{CIP1/WAF1}$ is sufficient to induce differentiation in Ls174T cells. Moreover, spontaneous differentiation of confluent CaCo-2 CRC cells, a model for enterocytic differentiation, involves the downregulation of β-catenin signaling and the upregulation of p21$^{CIP1/WAF1}$ (Evers et al., 1996; Yang et al., 2001a; Mariadason et al., 2001). In agreement with a central role of p21$^{CIP1/WAF1}$ in WNT cascade-driven tumorigenesis, a genetic link between *p21$^{CIP1/WAF1}$* and polyp formation in *APC* mutant mice has recently been reported (Yang et al., 2001b). The related CDK inhibitor p27^{KIP1} can also trigger differentiation in intestinal cell lines (Deschenes et al., 2001; Quaroni et al., 2000). Like for *p21$^{CIP1/WAF1}$*, a genetic link between *p27^{KIP1}* and polyp formation in *APC* mutant mice has been reported (Philipp-Staheli et al., 2002). Our experiments identify p21$^{CIP1/WAF1}$ as the primary CDK inhibitor mediating the differentiation process. However, the phenotype in the intestine of mice with targeted *p21$^{CIP1/WAF1}$* or *p27^{KIP1}* is

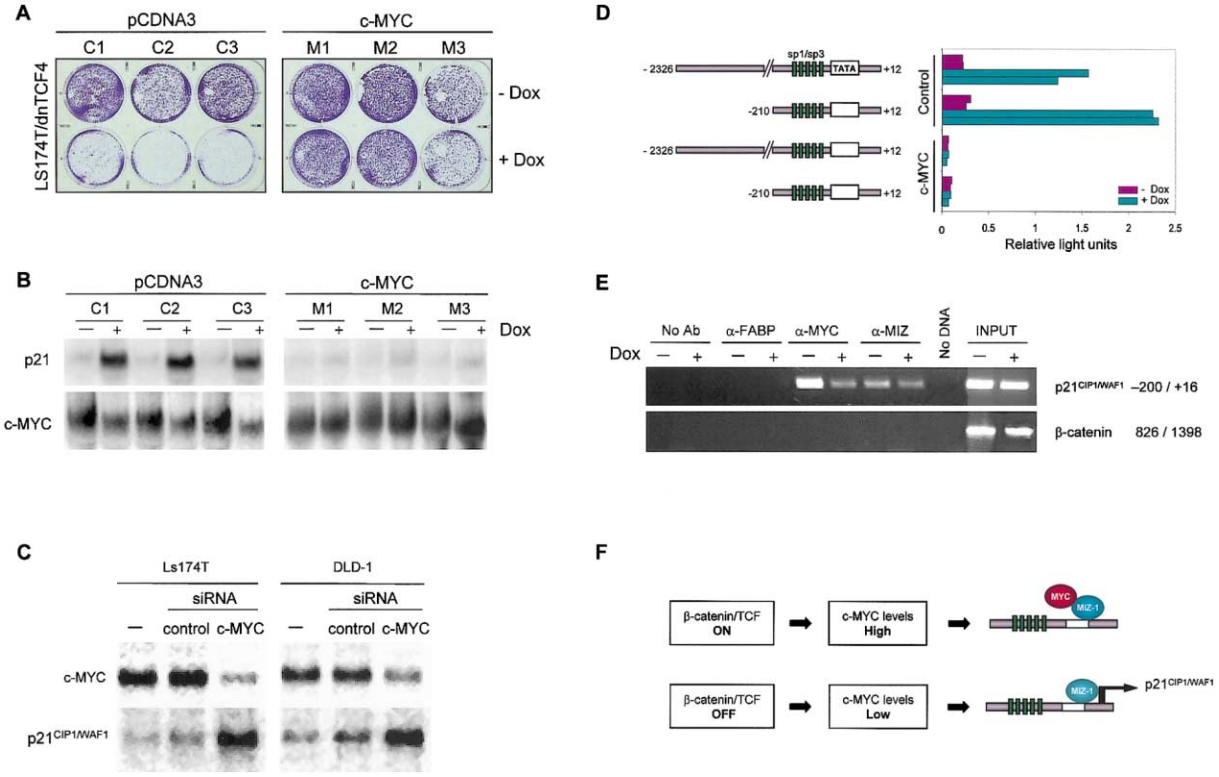

Figure 6. c-MYC Represses p21$^{CIP1/WAF1}$ Expression in Ls174T Cells

(A) Control Ls174T/dnTCF-4 cells (left) transfected with empty pCDNA3 vector stop proliferating upon induction of dnTCF-4 by doxycycline as the parental Ls174T/dnTCF-4 (Figure 1E). The same cells constitutively expressing c-MYC override the cell cycle arrest imposed by dnTCF-4 induction with doxycycline and continue to proliferate (right). Crystal violet staining of three independent control (C 1–3) or c-*MYC* transfectants (M 1–3) after 5 days with or without doxycycline are shown.

(B) *p21$^{CIP1/WAF1}$* mRNA (top) is not induced in Ls174T/dnTCF-4 constitutively expressing c-*MYC* (bottom) (M 1–3) upon induction with doxycycline. Strong upregulation is observed in control cells transfected with empty pcDNA3 (C 1–3).

(C) siRNA-mediated c-*MYC* downmodulation in Ls174T and in DLD-1 CRC cells results in upregulation of p21$^{CIP1/WAF1}$. c-*MYC* (top) and p21$^{CIP1/WAF1}$ (bottom) mRNA levels in nontransfected (−), transfected with control scrambled RNA duplexes (control), or with siRNA duplexes directed toward c-MYC (c-MYC) in the indicated cell lines.

(D) The region responsible for dnTCF-4-induced p21$^{CIP1/WAF1}$ upregulation in Ls174T cells maps to a 200 bp fragment in the proximal promoter containing five sp-1 sites, the TATA box, and the transcription initiation site. This fragment is not upregulated in Ls174T/dnTCF-4 constitutively expressing c-MYC. The depicted p21$^{CIP1/WAF1}$ promoter fragments were cloned in front of the Firefly Luciferase reporter and transiently transfected to Ls174T/dnTCF-4 cells with or without doxycycline. Renilla luciferase values were used as a transfection control.

(E) c-MYC and MIZ-1 antibodies both immunoprecipitate the 200 bp fragment responsible for p21$^{CIP1/WAF1}$ upregulation by ChIP assays (top). Unrelated antibodies fail to immunoprecipitate this sequence (α-FABP), and all antibodies fail to immunoprecipitate an unresponsive control (bottom).

(F) Diagram depicting the proposed mechanism for β-catenin/TCF control of p21$^{CIP1/WAF1}$ expression. In cells with active β-catenin/TCF complexes, high levels of c-MYC would bind to and inhibit MIZ-1 activation of the p21$^{CIP1/WAF1}$ promoter. Inhibition of β-catenin/TCF activity leads to a decrease in the levels of c-MYC, which no longer prevents c-MIZ-1 from activating p21$^{CIP1/WAF1}$ transcription.

grossly normal (Yang et al., 2001b; Nakayama et al., 1996; Fero et al., 1996; Kiyokawa et al., 1996). The two molecules may cooperate in this process in vivo. Such cooperation occurs between p21$^{CIP1/WAF1}$ and p57^{KIP2} during muscle differentiation (Zhang et al., 1999).

In conclusion, these data indicate that cell proliferation and differentiation are intimately coupled in intestinal cells and that β-catenin and TCF regulate these processes by controlling the levels of the key protein p21$^{CIP1/WAF1}$ in CRC cells. Our observations imply that TCF constitutes the dominant switch between the proliferating progenitor and the differentiated intestinal cell (see model in Figure 7). This is recapitulated in the CRC cells used in this study, despite the presence of multiple additional mutations in these cells. The current study validates the disruption of the β-catenin/TCF complex as a therapeutic strategy to revert the transformed phenotype in colorectal cancer.

Experimental Procedures

Cell Culture and Transfections

T-REx system (Invitrogen) was used according to manufacturer's instructions to generate dnTCF1, dnTCF-4, or p21$^{CIP1/WAF1}$ inducible CRC cell lines. Resistant colonies were tested for induction by immunocytochemical staining. Ls174T/dnTCF-4 constitutively expressing c-*MYC,* c-*MYB*, c-*ETS2*, *BMP4, ENC1*, p21$^{CIP1/WAF1}$ antisense, and control empty vector were derived by further transfecting these cells with pCDNA3 vector (Invitrogen) carrying the appropriate cDNA. 21-mer RNA duplexes (Dharmacon) directed against c-*MYC* sequence 5′-AACGTTAGCTTCACCAACAGG (bp 565–585) were transfected by

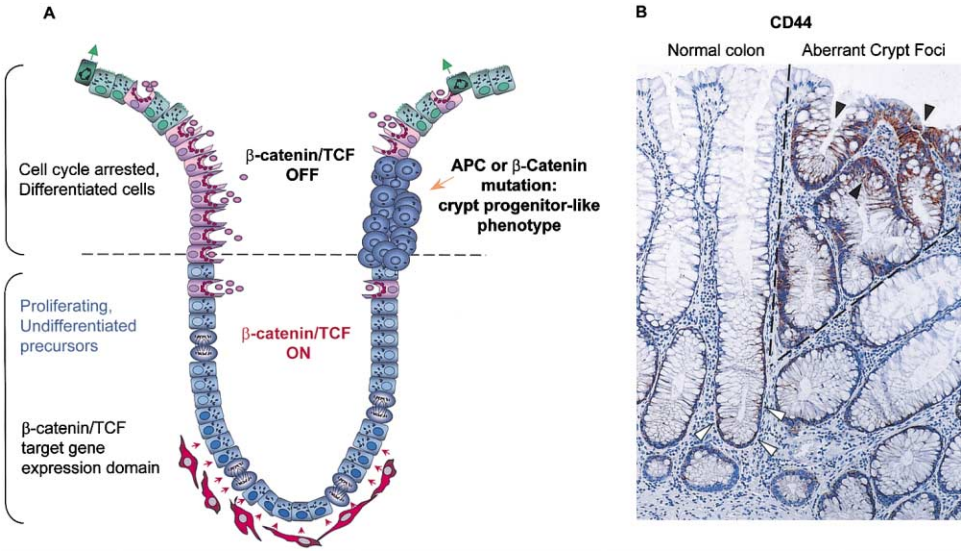

Figure 7. Model for the Role of β-Catenin/TCF in the Early Stages of Intestinal Tumorigenesis

(A) Schematic representation of a colon crypt and proposed model for polyp formation. At the bottom third of the crypt, the progenitor proliferating cells accumulate nuclear β-catenin. Consequently, they express β-catenin/TCF target genes. An uncharacterized source of WNT factors likely resides in the mesenchymal cells surrounding the bottom of the crypt, depicted in red. As the cells reach the midcrypt region, β-catenin/TCF activity is downregulated and this results in cell cycle arrest and differentiation. Cells undergoing mutation in *APC* or *β-catenin* become independent of the physiological signals controlling β-catenin/TCF activity. As a consequence, they continue to behave as crypt progenitor cells in the surface epithelium, giving rise to ACFs.

(B) CD44, a β-catenin/TCF target, exemplifies this model. It is expressed in the normal proliferative compartment at the bottom of the crypts (white arrowheads) and also in the early lesions arising at the surface epithelium (black arrowheads).

oligofectamine (Invitrogen) according to manufacturer's instructions.

Probe Preparation and Microarray Procedures

Fluorescent-labeled cDNA was prepared from 1 μg of poly(A) mRNA by oligo dT-primed polymerization using Superscript II reverse transcriptase in the presence of either Cy3- or Cy5-labeled dCTP as described (see http://cmgm.stanford.edu/pbrown/protocols.html). The appropriate Cy3- and Cy5-labeled probes were pooled and hybridized to microarrays in a volume of 25 μl under a 22 × 14 mm glass coverslip for 16 hr at 65°C and washed at a stringency of 0.2× SSC. The microarray contains 24,000 DNA spots representing approximately 10,000 known full-length cDNAs and 14,000 ESTs of clones made available by Research Genetics (see Supplementary Data at http://www.cell.com/cgi/content/full/111/2/241/DC1).

Fluorescent images of hybridized microarrays were obtained using a genepix 4000 microarray scanner (Axon Instruments, Inc). Images were analyzed with scanalyze or with genepix 3.0. Fluorescence ratios were stored in a custom database. Fluorescent ratios were calibrated independently for each array by applying a single scaling factor to all fluorescent ratios from each array; this scaling factor was computed so that the median fluorescence ratio of the measured spots on each array was 1.0. We selected genes represented by good-quality spots for which the fluorescent intensity in each channel was greater than 1.5 times the local background.

Cell Cycle Analysis

3×10^6 (Ls174T) or 10^6 (DLD-1) cells were seeded in 9 cm dishes and doxycycline was added (1 μg/ml). After 20 hr, BrdU (Roche) was added for 20 min. Cells were then fixed in ethanol 70%. Nuclei were isolated and incubated with α-BrdU-FITC (BD), and cell cycle profiles were determined by FACS analysis. Crystal violet staining on methanol-fixed cells was done on cells after 5 days induction.

Immunohistochemistry

Antibodies used: EPHB2 and EPHB3 from R&D systems; BMP4 from Novacastra; ENC1 from Pharmingen; c-MYB from Santa Cruz Biotechnology; $p21^{CIP1/WAF1}$ from Pharmingen; carbonic anydrase II from Rockland; and β-catenin from Transduction Laboratories. TCF1 and TCF-4 antibodies were described elsewhere (Barker et al., 1999; Castrop et al., 1995). Immunostainings were performed according to standard procedures. Envision+ kit (DAKO) was used as a secondary reagent. Stainings were developed using DAB (brown precipitate). Slides were counterstained with hematoxylin.

Chromatin Immunoprecipitation (ChIP)

ChIP analysis was performed as described previously (Staller et al., 2001). Immunoprecipitations were carried out with anti-MYC (Upstate) and anti-MIZ antibodies overnight at 4°C. The oligonucleotide sequences used to amplify the DNA fragments are available upon request.

Reporter Assays

The sequence of the $p21^{CIP1/WAF1}$ promoter (el Deiry et al., 1995; Tetsu and McCormick, 1999) was cloned in pGL3 (Promega). Transient transfections of the appropriate Firefly luciferase reporters and Renilla luciferase as a transfection control were performed with Fugene 6 (Roche) and measured with the Dual Luciferase Reporter Assay System (Promega).

Acknowledgments

We thank Patrick Brown for making DNA microarrays and support services available for the DNA microarray studies. E.S. and E.B. are recipients of Marie Curie Fellowships. This work was supported in part by a grant from the NCI to P.O. Brown.

Received: February 4, 2002
Revised: August 29, 2002

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