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Transcription Factor PROX1 Induces Colon Cancer Progression by Promoting the Transition from Benign to Highly Dysplastic Phenotype

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SUMMARY

The *Drosophila* transcription factor Prospero functions as a tumor suppressor, and it has been suggested that the human counterpart of Prospero, PROX1, acts similarly in human cancers. However, we show here that PROX1 promotes dysplasia in colonic adenomas and colorectal cancer progression. PROX1 expression marks the transition from benign colon adenoma to carcinoma in situ, and its loss inhibits growth of human colorectal tumor xenografts and intestinal adenomas in *Apc*$^{min/+}$ mice, while its transgenic overexpression promotes colorectal tumorigenesis. Furthermore, in intestinal tumors PROX1 is a direct and dose-dependent target of the β-catenin/TCF signaling pathway, responsible for the neoplastic transformation. Our data underscore the complexity of cancer pathogenesis and implicate PROX1 in malignant tumor progression through the regulation of cell polarity and adhesion.

INTRODUCTION

Colorectal cancer (CRC) is the second most common type of malignancy in the Western world. In CRC development, a preneoplastic polyp gradually accumulates genetic changes that allow uncontrolled proliferation and cell survival, followed by invasive and metastatic properties typical of carcinoma. Activation of the APC/β-catenin/TCF pathway is an initiating event in the majority of human CRCs (reviewed in Kinzler and Vogelstein, 1996; Weinberg, 2007). The APC protein binds cytoplasmic β-catenin targeting it for degradation. If the degradation is inhibited, for example, following Wnt signals, β-catenin accumulates in the

SIGNIFICANCE

Understanding the mechanisms that control tumor progression beyond the initiating events, such as activation of TCF/β-catenin signaling in colorectal cancer, is important for the development of cancer therapies. Here we show that the transcription factor PROX1, previously shown to play an essential role in the development of liver, lymphatic vasculature, and eye, is an important regulator of progression from a benign to a highly dysplastic phenotype in colorectal tumors. This result uncovers a cancer- and tissue-specific effector of the TCF/β-catenin cascade and suggests that PROX1 is a potential target for the development of colon cancer therapy.

nucleus, where it forms a complex with the TCF transcription factors to control gene expression. Familial adenomatous polyposis patients, who have *APC* mutations that block β-catenin degradation, develop hundreds of intestinal polyps and progress to CRC by the age of 40. At least one *APC* allele is mutated in about 60% of sporadic CRCs, and somatic β-catenin mutations are found in 50% of CRCs that have wild-type *APC* alleles.

An immediate consequence of loss of *Apc* in the normal mouse intestinal epithelium is the expansion of the progenitor cell population, in agreement with the in vitro studies showing that the β-catenin/TCF pathway controls colon cancer cell proliferation and expression of progenitor cell-specific genes (Sansom et al., 2004; van de Wetering et al., 2002). The C-myc transcription factor, which is a direct target of β-catenin/TCF, is largely responsible for the increased cell proliferation and apoptosis and loss of cell migration and differentiation after *Apc* deletion (Sansom et al., 2007). However, in humans, several years are required for the complex multistage progression of APC mutant tumors from benign adenoma to carcinoma in situ and finally to metastatic cancer. Knowledge of the molecular regulation of these transitions is still incomplete. Here we show that the homeobox transcription factor PROX1 is an intestinal-specific target of the β-catenin/TCF pathway and that it is essential for the transition from benign to severe dysplasia, which is associated with a high risk of subsequent colon carcinoma.

PROX1 is an evolutionarily conserved transcription factor that controls neural precursor cell development in *Drosophila*, and lymphatic endothelial and retinal progenitor cell differentiation and the development of the liver and lens in mice (Chu-Lagraff et al., 1991; Dyer et al., 2003; Oliver et al., 1993; Sosa-Pineda et al., 2000; Wigle et al., 1999; Wigle and Oliver, 1999). The *Drosophila* counterpart of PROX1, *Prospero*, acts as a brain tumor suppressor by preventing neuroblast self-renewal (Betschinger et al., 2006). It has recently been suggested that PROX1 has a similar role in human cancers. Decreased PROX1 levels were observed in hepatocellular carcinomas and biliary duct cancers, concomitantly with loss of heterozygosity and epigenetic silencing of *PROX1* (Laerm et al., 2007; Shimoda et al., 2006). We have investigated the expression of PROX1 in other common human cancers and noticed that it is overexpressed in the majority of CRCs. Here we explore this observation and report findings showing that PROX1 is a target of the β-catenin/TCF pathway in CRC, where it promotes dysplasia, tumor growth, and malignant progression.

RESULTS

PROX1 Is Overexpressed in Human CRC

In a cancer gene profiling array, which contains cDNAs representing 241 human cancers and corresponding normal tissues, PROX1 mRNA was significantly increased in 35 of 53 CRC samples, but not in breast, uterine, lung, kidney, ovarian, or thyroid tumors (Figures 1A and 1C). PROX1 is a marker for lymphatic vessels, which are abundant in normal colonic submucosa and around colon carcinomas. However, hybridization with a probe for the lymphatic endothelial hyaluronan receptor LYVE-1 indicated that the increased expression of PROX1 in CRC cannot be attributed to the lymphatic vessels in the tumors (Figure 1B). PROX1 mRNA was also present in the CRC cell lines SW480, SW48, COLO205, LS174T, and HT-29, but not in the cell lines DLD1, WiDr, HCT-116, or SW403, or 18 other non-CRC cell lines (Figure S1 available online). PROX1 cDNA from SW480, COLO205, and LS174T cells did not contain coding region mutations, suggesting that PROX1 retains its wild-type function (H. Kubo, personal communication).

Immunohistochemical staining revealed increased PROX1 expression in 13/16 adenomas and in 16/24 adenocarcinomas, while only rare scattered PROX1-positive cells were observed in normal colonic epithelium or in hyperplastic polyps (Figure S2 and data not shown). High PROX1 levels were observed in the majority of cells in nine adenomas and in six carcinomas, whereas in the other lesions heterogeneous expression of PROX1 occurred. In four carcinoma samples no staining for PROX1 was seen, except in the lymphatic vessels (data not shown).

In colon adenoma samples containing both low- and high-grade dysplasia (n = 5), the highest levels of PROX1 were observed in the areas of severe dysplasia, characterized by disruption of the glandular architecture, crowding, and prominent cellular atypia (Figures 1D and 1E). PROX1-positive cells were also strongly positive for nuclear β-catenin, a hallmark of activated Wnt signaling. Notably, we observed glands that contained small clusters or isolated PROX1 and nuclear β-catenin-positive cells along with areas that were almost entirely positive for both of these proteins, suggesting that these glands are undergoing clonal selection toward a highly dysplastic phenotype (Figure 1F).

The tumor suppressor p53 is commonly mutated in CRC, and mutated p53 is overexpressed in highly dysplastic adenomas (Saleh et al., 1998). Although PROX1 and p53 were located in overlapping sets of cells in the highly dysplastic regions (Figure S2), isolated clusters of Prox1 and nuclear β-catenin-positive cells expressed only low levels of p53, suggesting that PROX1 upregulation occurs prior to p53 mutation (Figure S2). Collectively, the data suggested that PROX1 upregulation defines the transition from adenoma to carcinoma in situ and that it plays a role in the β-catenin/TCF-dependent progression of colon cancer.

Prox1 Is Overexpressed in Mouse Models of Intestinal Cancer with Activated TCF/β-Catenin Signaling

Azoxymethane (AOM) induces colon cancer in rodents through the development of microscopic lesions, aberrant crypt foci (ACFs), which further progress to colon adenomas and carcinomas (Boivin et al., 2003). These neoplasms are morphologically similar to human CRC, including abnormal activation of the β-catenin/TCF pathway (Sheng et al., 1998). In a genetic model of intestinal neoplasia, the $Apc^{min/+}$ mice carry a germline mutation in *Apc*, which, together with somatic inactivation of the remaining wild-type allele, leads to abnormal β-catenin/TCF signaling and development of multiple polyps in the small intestine (Su et al., 1992).

We observed high Prox1 levels in intestinal neoplasms of both AOM-treated and $Apc^{min/+}$ mice (Figures 2A–2C and Figure S3). Prox1 was present in tumor cells with high cytoplasmic and nuclear β-catenin, but not in cells of normal glands with membrane localization of β-catenin (Figures 2D–2G). Notably, Prox1 expression was heterogeneous in small adenomas of the $Apc^{min/+}$ mice

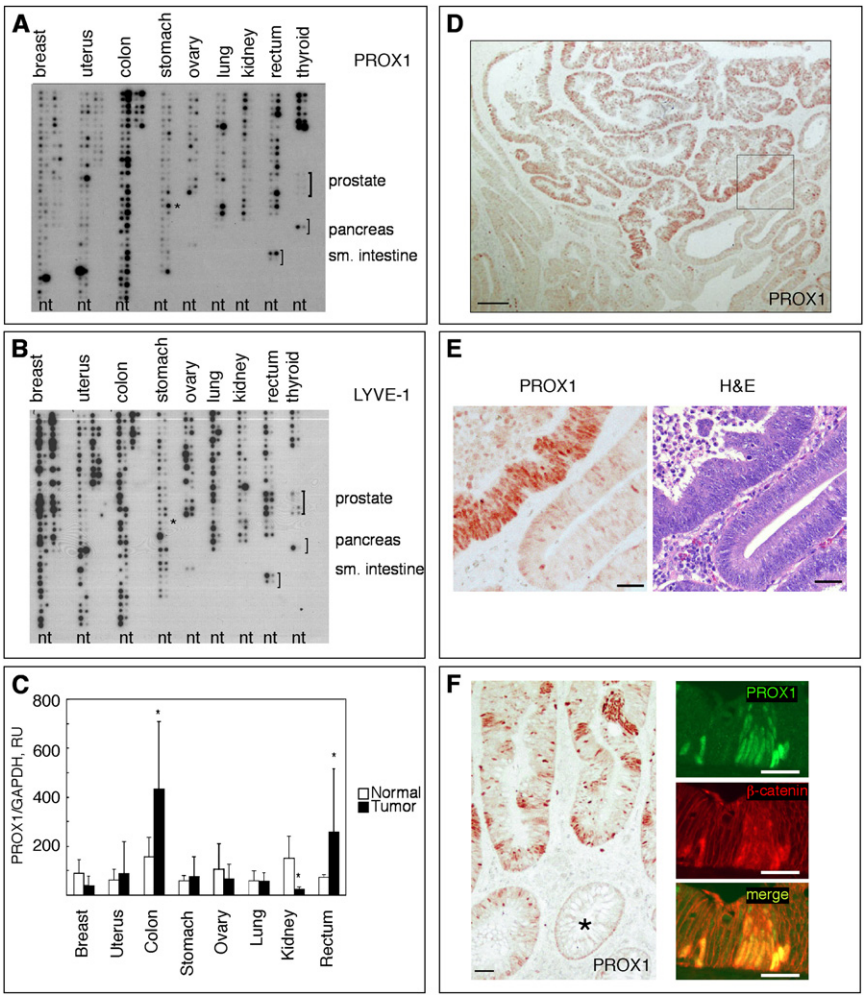

Figure 1. PROX1 Is Overexpressed in Human Colorectal Cancer and Severe Dysplasia

(A and B) A cancer gene-profiling array was hybridized to probes for PROX1 (A), and the lymphatic endothelial marker LYVE-1 (B).

(C) Quantification of the dot blot in (A); data are normalized to GAPDH levels from the same blot and presented as mean ± SD. RU, relative units. Asterisks in (A) and (B) indicate a colon sample. Asterisks in (C) indicate samples in which PROX1 expression is significantly different from that of the normal tissue ($p < 0.05$).

(D) PROX1 is induced upon transition to carcinoma in situ. Staining for PROX1 (brown) of colon polyp harboring both low- and high-grade dysplasia.

(E) High-power magnification of the inset in (D) and adjacent section stained with hematoxylin and eosin shows severely disturbed glandular architecture in PROX1-positive area.

(F) PROX1-positive cells contain high levels of cytoplasmic and nuclear β-catenin. Asterisk, normal colonic gland.

Scale bars, 100 μm in (D), 25 μm in (E) and (F).

and high in large adenomas, suggesting that Prox1-expressing cells have a selective growth advantage (Figure S3). Interestingly, while all Prox1-positive cells were positive for nuclear β-catenin, some cells with high nuclear β-catenin did not express Prox1, suggesting that additional factors are necessary for its expression, or that the degree of activation of the TCF/β-catenin pathway varies even in the presence of abundant nuclear β-catenin.

Mutations in components of the TGFβ pathway, such as *TGFBRII* and *SMAD4*, occur in human CRC, and inactivation of the TGFβ1-binding protein Ltbp4 leads to CRC in mice (Sterner-Kock et al., 2002). However, Prox1 was absent from colonic adenocarcinomas of *Ltpb4*$^{-/-}$ mice, which had a normal distribution of β-catenin (Figure S3 and data not shown). Similarly, we did not observe Prox1 overexpression in gastric adenomas from intestinal trefoil factor 1-deficient mice (data not shown). These results suggested that Prox1 is located downstream of the APC/β-catenin/TCF pathway in vivo.

PROX1 Is Induced in Nascent Microadenomas but Not in the Expanded Progenitor Cell Population following Somatic Deletion of Apc

To pinpoint at what stage of tumor development Prox1 expression is activated, we used a mouse model where deletion of *Apc* in intestinal epithelium is induced by β-naphtoflavone. Loss of *Apc* leads to a rapid and drastic expansion of the progenitor cell compartment in the crypts, followed by formation of widespread micro and macroadenomas (Sansom et al., 2004). After 5 days of β-naphtoflavone treatment, Prox1 was not induced in expanded proliferating crypts, instead it was present in the dysplastic epithelium that began to outpocket into the surrounding stroma and to form microadenomas (Figures 2H and 2J). Strikingly, Prox1 was expressed in the majority of highly dysplastic epithelial cells 6 days after induction (Figure 2I). These data further confirm that Prox1 lies downstream of the activated TCF/β-catenin pathway and show that either additional signals or prolonged and very high levels of TCF/β-catenin signaling are necessary for Prox1 induction in the initiated progenitor cells. Most importantly, together with the high levels of Prox1 in *Apc*-deficient macroadenomas and human colon carcinomas in situ, these results implicate Prox1 in tumor establishment, maintenance, and/or progression rather than in the regulation of normal progenitor cell phenotype or cancer initiation.

Prox1 Is Expressed in a Subset of Enteroendocrine Cells and Their Progenitors in Normal Intestinal Epithelium

Intestinal epithelium is composed of slowly dividing stem cells located at the bottom of the crypts, the transient amplifying cells, which give rise to the enterocytes, goblet, enteroendocrine, and Paneth cells, and terminally differentiated cells, located in the luminal part of the colon or villi in the small intestine. In mouse intestine Prox1 was present in scattered cells in the villi and crypts (Figure 3A). Enteroendocrine cells that expressed hormones PYY, cholecytokinin (CCK), and glucagon-like peptide-1 (GLP-1)

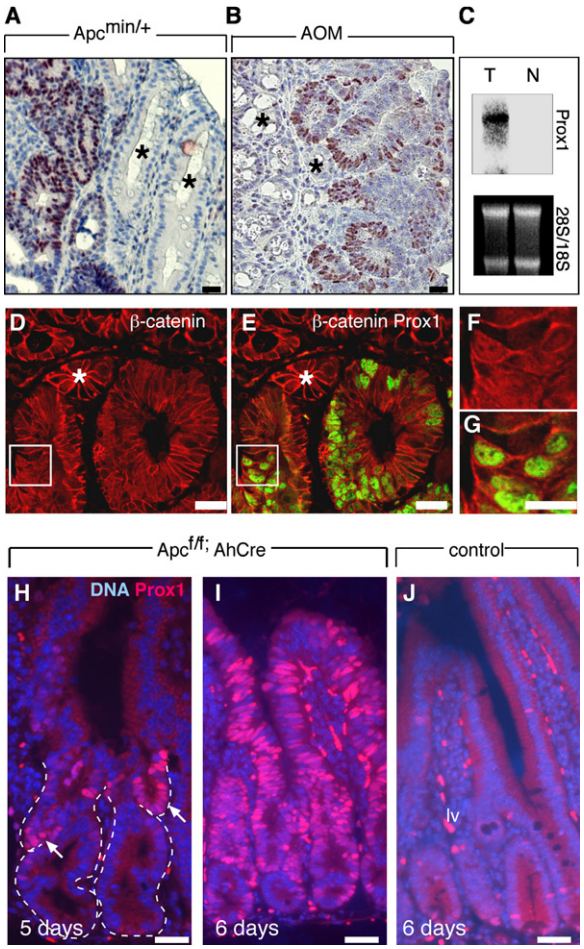

Figure 2. Prox1 Is Overexpressed in Mouse Tumors with Activated TCF/β-Catenin Signaling

(A and B) Prox1 (brown nuclear staining) is expressed in colonic neoplasms from *Apc*$^{min/+}$ and AOM-treated mice.

(C) High Prox1 mRNA levels in *Apc*$^{min/+}$ adenoma. T, tumor; N, normal.

(D) Increased cytoplasmic and nuclear β-catenin (red) in AOM-induced adenoma. Asterisk, normal intestinal gland.

(E) Double staining of AOM-induced lesion for β-catenin (red) and Prox1 (green).

(F and G) Prox1 is present in tumor cells with high nuclear β-catenin. High-power view of the white squares in (D) and (E).

(H–J) Prox1 is induced in dysplastic epithelial cells (arrows in [H]) but not the expanded progenitor population following immediate *Apc* deletion. Staining for Prox1 (red) in small intestine of *Apc*$^{flox;flox}$; *Ah*Cre and control mice after 5 and 6 days of treatment with β-naphtoflavone. lv, Prox1-positive lymphatic vessels.

Scale bars, 20 μm in (A) and (B), 10 μm in (D)–(G), 25 μm in (H)–(J).

frequently coexpressed Prox1 (range 40%–80%), while serotonin-expressing cells were only rarely Prox1-positive (<10%) (Figures 3C–3E and data not shown). In addition, a subset of Prox1-positive cells in the crypts expressed enteroendocrine progenitor marker neurogenin-3 (Ngn3), but not the Paneth cell marker lysozyme (Figures 3C–3E). Unlike Prox1-positive cells in intestinal adenomas, Prox1-positive cells in the normal gut did not proliferate, as determined by BrdU uptake or staining for phosphohistone H3 (Figure 3B and data not shown). In agreement with these data, Prox1-positive cells were absent from the intestinal epithelium of neonatal *Ngn3*$^{-/-}$ mice, which lack enteroendocrine cells (Jenny et al., 2002; Figures 3F and 3G). A similar expression pattern was observed in human colon and small intestine, where nonproliferating PROX1-positive cells were found to reside both in the crypt and in differentiated compartment, and a subset of cells expressed the neuroendocrine marker chromogranin A (Figures 3H–3J). The data show that in the normal intestinal epithelium Prox1 is mainly produced in a subset of mature enteroendocrine cells as well as either in Ngn3+ enteroendocrine precursors undergoing terminal differentiation or in long-lived enteroendocrine progenitors (Bjerknes and Cheng, 2006).

The β-Catenin/TCF Pathway Controls PROX1 Expression in CRC

The CRC cell line SW480, which we found to be PROX1 positive (Figure S1), contains two cell populations: epithelial-like slowly growing adherent cells (A) and weakly adherent rounded cells (R), which form aggressive tumors and are resistant to vitamin D-induced growth arrest and differentiation (Palmer et al., 2001; Tomita et al., 1992). PROX1 was strongly expressed only in the R cells (Figure S4). We isolated several clones of SW480R PROX1-positive and SW480A PROX1-negative cells and confirmed their common origin using microsatellite analysis (Figure S4 and Supplemental Experimental Procedures). As expected (Palmer et al., 2001; Tomita et al., 1992), SW480R but not SW480A cells formed rapidly growing tumors when implanted subcutaneously in *nu/nu* mice (0.73 ± 0.3 g versus 0.03 ± 0.01, respectively, $n = 6$, $p < 0.05$ at 28 days). Moreover, when a 50%–50% mixture of SW480R and SW480A cells was implanted, only PROX1-positive SW480R cells were recovered from the subsequent tumors, demonstrating that these cells have a selective growth advantage in vivo (data not shown). The gene expression profile of SW480R versus SW480A cells correlated with the aggressive and adhesion-independent tumor phenotype. Notably, SW480R cells lacked many components of the actin, intermediate filament, and microtubule networks, such as gelsolin, filamins A and B, ezrin, moesin, vimentin, integrins, and tubulins (Table S1 and data not shown); they expressed higher levels of the proto-oncogene CMET and the receptor tyrosine kinase FGFR4, which is associated with malignant transformation in colorectal carcinomas (Bange et al., 2002) and had lower levels of cell-cycle inhibitor CDKN1a (p21$^{CIP1/WAF1}$) and metalloprotease inhibitor TIMP-3 (Figure S4).

Because the activation of β-catenin/TCF signaling correlated strongly with PROX1 expression in the in vivo samples, we studied the relationship of PROX1 with this pathway. Suppression of PROX1 in SW480R cells using two different siRNAs did not affect β-catenin/TCF-responsive reporter activity, the nuclear localization, or the levels of active, nonphosphorylated β-catenin (Figure S5 and Figure 4A). In contrast, suppression of β-catenin resulted in almost complete loss of PROX1 expression (Figure 4A). Suppression of PROX1 was also observed in SW480R cells transfected with a dominant-negative mutant of TCF4, which disrupts β-catenin/TCF-mediated transcription (Figure 4B). However, p21$^{CIP1/WAF1}$ overexpression that induces growth arrest and differentiation of CRC cells (van de Wetering et al., 2002) did not alter PROX1 levels (Figure 4B). Taken

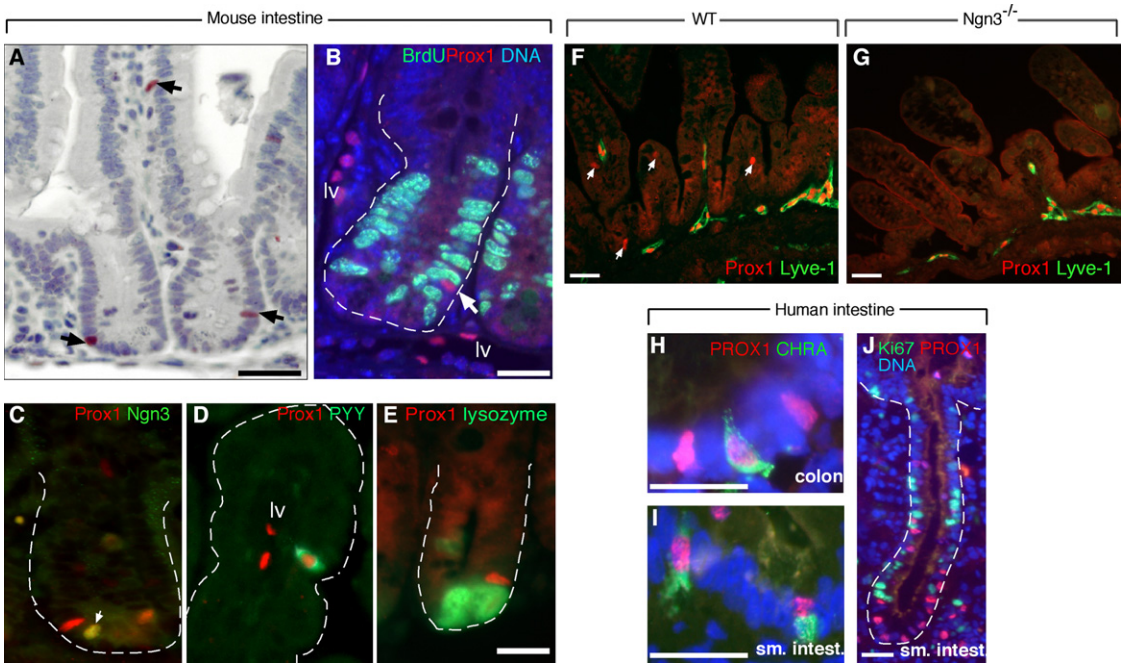

Figure 3. PROX1 Expression in Normal Intestinal Epithelium

(A) Prox1-positive cells (brown, arrows) are present in the crypts and villi in mouse small intestine.

(B) Prox1-positive cells in the crypt (red, arrow) are located below the proliferation zone, identified by staining for BrdU after 120 min pulse labeling (green). lv, lymphatic vessel.

(C–E) Staining for Prox1 (red) and Ngn3, PYY, or lysozyme (green).

(F and G) Prox1 expression is lost in duodenal epithelium of *Ngn3*$^{-/-}$ mice but maintained in lymphatic vessels of *Ngn3*$^{-/-}$ and control wild-type mice. Staining for Prox1 (red) and the lymphatic marker LYVE-1 (green).

(H and I) Some PROX1-positive cells (red) in human colon (H) and small intestine (I) express chromogranin A (green).

(J) Prox1-positive cells (red) are located below the Ki67+ proliferation zone (green) in human small intestine (only an intestinal crypt is shown).

Nuclei are stained blue with Hoechst 33342 in (B) and (H)–(J). Scale bars, 25 μm.

together with the in vivo expression data, these results show that PROX1 lies downstream of β-catenin/TCF signaling.

Although β-catenin/TCF signaling was necessary for PROX1 expression in the SW480R cells, PROX1 levels were low in SW480A cells carrying the same inactivating mutation of *APC*. As the SW480R cells displayed a two-fold higher activation of the TCF-responsive promoter TopFLASH and higher levels of both total and active β-catenin (Figure 4C), we hypothesized that PROX1 expression is induced only in cells with high levels of β-catenin/TCF activity. Indeed, transfection of SW480A cells with constitutively active LEF1-β-catenin fusion construct increased TopFLASH reporter activity 11.3 ± 2.9-fold in comparison to GFP-transfected cells. Furthermore, overexpression of LEF1-β-catenin induced PROX1 in transfected cells, while overexpression of GFP had no effect (Figure 4D, 38% versus 1% PROX1-positive transfected cells in two independent experiments). These findings suggested that only high levels of β-catenin/TCF activation induce PROX1 in CRC.

To determine if *PROX1* is a direct target of TCF/β-catenin, we searched the *PROX1* promoter/enhancer regions for evolutionary conserved TCF/LEF binding sites (Hallikas et al., 2006). Analysis of human, rat, and mouse sequences identified two conserved TCF/LEF binding sites at −43 kb and −49 kb relative to the human *PROX1* start codon (Figure S6). Electrophoretic mobility shift assay experiments with nuclear extracts from SW480R cells and DNA oligonucleotide sequences from PROX1 enhancer regions confirmed specific affinity only to the −49 kb TCF/LEF binding site, as shown by the competition and supershift experiments (Figure 4E and data not shown). Furthermore, in luciferase reporter assays, cotransfection with LEF-1-β-catenin fusion protein strongly increased only the activity of the −49 kb-luc fragment (Figure S5). Finally, using chromatin immunoprecipitation (ChIP) we showed that endogenous TCF4 binds specifically to the −49 kb element in SW480R cells, while no interaction was observed in PROX1-negative SW480A and HCT116 cells (Figure 4F). At the same time, TCF4 interacted with TCF/LEF site in *CMYC* promoter in all three cell types, in agreement with previous reports (Beiter et al., 2005). These results suggested that *PROX1* transcription is directly regulated by TCF/β-catenin signaling through the TCF/LEF binding sites of the −49 kb PROX1 element. In PROX1-negative CRC cells, this element may be inaccessible to TCF/LEF/β-catenin, while becoming available via further increased TCF/β-catenin signaling (Figure 4D). Although alternative explanations are possible, we suggest that prolonged exposure of progenitor cells to TCF/β-catenin signaling, as observed in the *Apc*$^{flox/flox}$;*Ah*-Cre mice, predisposes them for further epigenetic changes facilitating access to the −49 kb enhancer and leading to Prox1 expression in nascent adenomas.

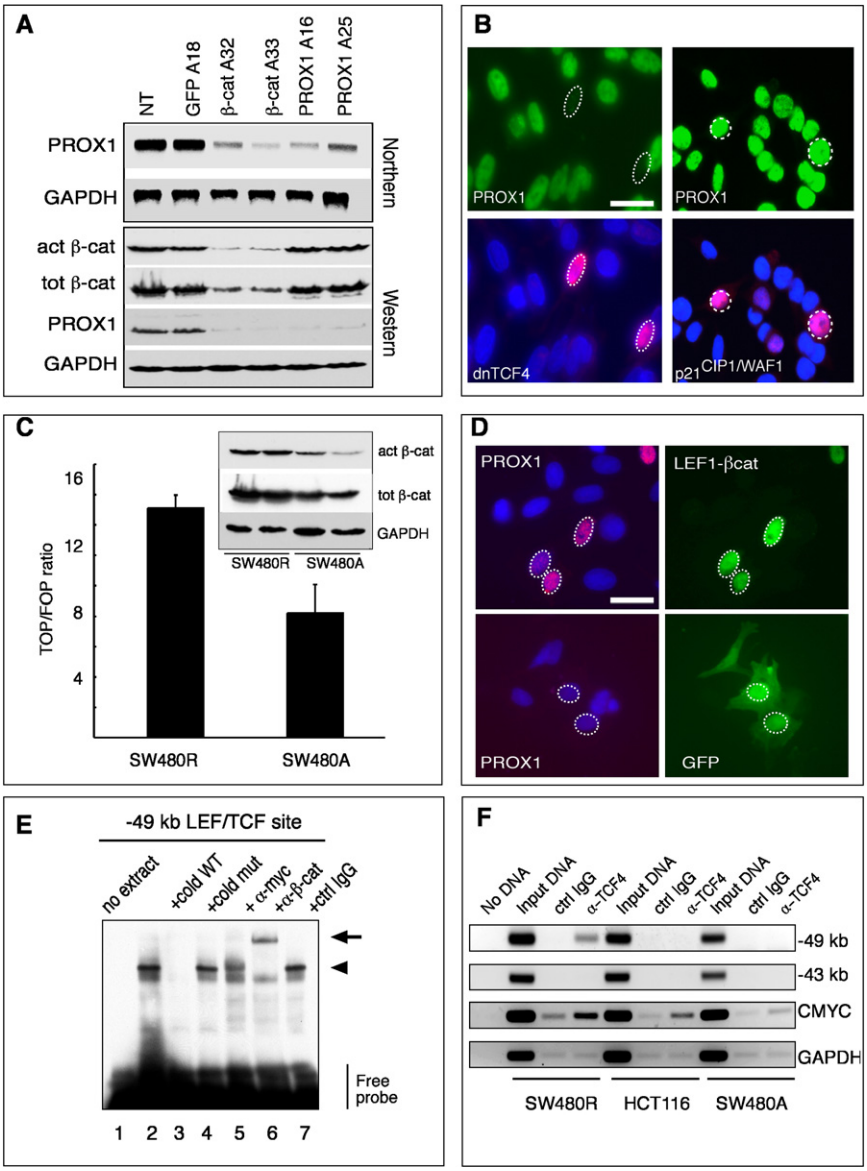

Figure 4. High Levels of β-Catenin/TCF Signaling Are Necessary for PROX1 Expression in CRC Cells

(A) Loss of β-catenin inhibits PROX1 expression in CRC cells. Northern and western blots of SW480R cells transfected with GFP siRNA A18, β-catenin siRNAs A32 and A33, or PROX1 siRNAs A16 and A25, or left untreated (NT).

(B) Dominant-negative TCF4 (dnTCF4) but not $p21^{CIP1/WAF1}$ suppresses PROX1 in SW480R cells. Staining for dnTCF4 or $p21^{CIP1/WAF1}$ (red), PROX1 (green), and DNA (blue).

(C) SW480R cells display higher TOPFlash reporter activity and active and total β-catenin than SW480A cells. Data in the graphs are shown as mean ± SD.

(D) Increased β-catenin/TCF signaling induces PROX1. Staining for PROX1 (red) in SW480A cells transfected with LEF1-β-catenin or GFP (green).

(E) Electrophoretic mobility shift assay of the −49 kb enhancer region of PROX1 containing TCF/LEF binding site. Nuclear extracts were from SW480R cells transfected with myc-tagged LEF1-β-catenin fusion construct. 1, no extract; 2, wild-type (WT) sequence; 3, competition with unlabeled WT sequence; 4, competition with unlabeled TCF/LEF site mutant sequence; 5, supershift with anti-myc antibody; 6, supershift with β-catenin antibody (arrow); 7, supershift with control mouse antibody.

(F) Detection of occupancy of the −49 and −43 kb *PROX1* enhancers by TCF4. Chromatin immunoprecipitation from SW480R, SW480A, and HCT116 cells with TCF4-specific or control antibodies. PCR for the indicated genomic regions; CMYC promoter region, known to bind TCF4 (Beiter et al., 2005), and exon 6 of GAPDH served as positive and negative controls.

Scale bars, 25 μm.

TCF/β-Catenin-Independent Prox1 Expression in Liver Cancer

Stabilizing mutations of β-catenin are frequently found in human liver cancer, and loss of *Apc* leads to liver tumors in mice (Colnot et al., 2004). On the other hand, decreased expression of PROX1 was recently reported in advanced liver cancer (Shimoda et al., 2006). Although the hepatocellular carcinoma HepG2 cells contain mutated β-catenin and high levels of PROX1 (de La Coste et al., 1998; and this study), dnTCF4 transfection did not affect their PROX1 expression, indicating that TCF/β-catenin does not control PROX1 in these cells (Figure 5A). Because the Ah promoter is active also in hepatocytes (Ireland et al., 2004), we could confirm that loss of *Apc* in hepatocytes of *Ah*-Cre; $Apc^{flox/flox}$ mice did not increase Prox1 expression, in spite of the nuclear translocation of β-catenin (Figures 5B and 5C). Thus, in response to activated TCF/β-catenin signaling, PROX1 expression is triggered only in a specific cellular context, such as in CRC, and this pathway does not control PROX1 expression in normal or transformed hepatocytes.

PROX1 Controls Cell Adhesion Program in CRC

PROX1 was previously reported to control the proliferation of lens fiber cells and the exit of retinal progenitor cell from the cell cycle (Dyer et al., 2003; Wigle et al., 1999). To understand the role of PROX1 in CRC, we studied the transcriptional profile of SW480R cells 48 hr after *PROX1* gene silencing with two *PROX1* siRNAs (Figure 6A and Figure S7). Using a false discovery rate of <0.05 and 2-fold change as cut-off values, 117 genes were upregulated and 31 downregulated following loss of PROX1 (Table S2 and Figure 6A).

Analysis of pathways induced upon *PROX1* suppression demonstrated strong enrichment for Gene Ontology (GO) categories ($p < 10^{-5}$): receptor activity, receptor binding, integral to plasma membrane, tissue development, regulation of signal transduction, intrinsic to plasma membrane, cyclic nucleotide metabolic process, actin binding, intercellular junction, and biological adhesion (Table S3 and Figure S8), suggesting that suppression of cell adhesion and regulation of actin cytoskeleton are the primary responses controlled by PROX1. Downregulated genes were enriched in GO terms protein translation, cell metabolism,

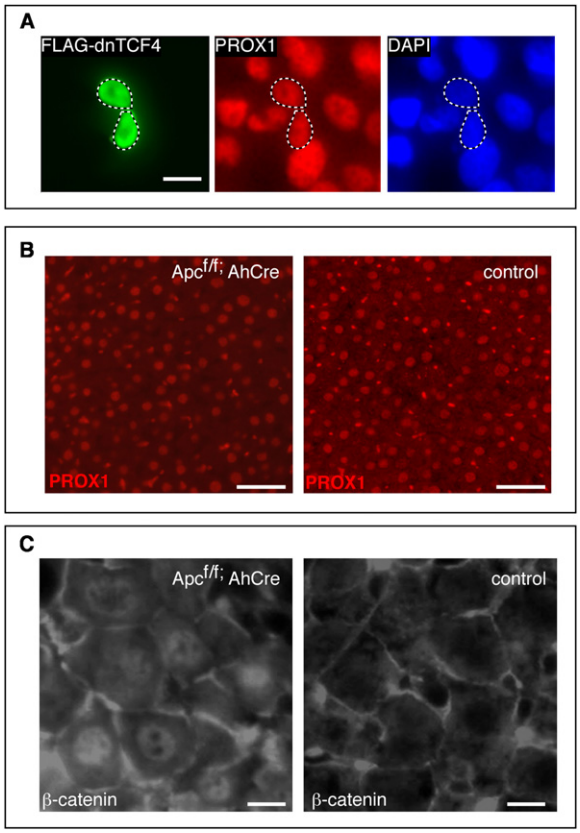

Figure 5. Prox1 Is a Tissue-Specific Target of the β-Catenin/TCF Pathway

(A) dnTCF4 does not repress PROX1 in HepG2 cells. Staining for dnTCF (green), PROX1 (red), and DNA (blue).

(B) Comparable levels of Prox1 in livers of *Ah*-Cre; $Apc^{flox/flox}$ and control mice after 6 days of β-naphtoflavone treatment.

(C) Nuclear translocation of β-catenin in hepatocytes of β-naphtoflavone-treated *Ah*-Cre; $Apc^{flox/flox}$ but not control mice.

Scale bars, 10 μm in (A), 50 μm in (B), and 10 μm in (C).

oxidative phosphorylation, and electron transport, confirming the transition to a more quiescent resting state (Table S3 and Figure S8). Despite the evident coregulation of many genes important in the cell-cell and cell-ECM interactions, there were no significant changes in the expression of genes associated with the regulation of cell cycle at 48 hr (Figure 6B), suggesting that loss of PROX1 does not directly affect cell proliferation. Loss of PROX1 in SW480R cells also shifted the transcriptional phenotype toward that of SW480A cells (Table S4).

In line with the upregulation of cell-adhesion pathways, *PROX1* suppression resulted in a morphological change by 72 hr posttransfection, which persisted for at least 10 days (Figure 6C). First, *PROX1* siRNA-transfected cells became more elongated and displayed extensive membrane ruffling. Later on they started to spread and adhere on the plate, and an increased number of actin stress fibers could be visualized in the cells (Figure 6C). At 120 hr posttransfection the *PROX1* siRNA-transfected cells proliferated at a lower rate than the GFP siRNA-transfected cells (BrdU-positive cells: 22% ± 0.5% *PROX1* siRNA A16, 18% ± 1% *PROX1* siRNA A25 versus 34% ± 4% GFP siRNA), and they expressed higher levels of cell-cycle inhibitor $p21^{CIP1/WAF1}$ (Figure S7). As changes in other gene categories preceded this event, we believe that decreased cell proliferation is secondary to the acquisition of a more adhesive phenotype upon loss of *PROX1*.

Growth of SW480R cells is adhesion independent, which is a hallmark of malignant transformation. Upregulation of cell adhesion upon loss of *PROX1* suggested that the cells reacquired the dependence on the signals from the extracellular environment. Indeed, *PROX1* siRNA-transfected cells were not able to form colonies in soft agar, which provides an adhesion-independent milieu, while the cells transfected with *GFP* siRNA and untransfected controls formed a similar number of colonies (Figure 6D). Thus, loss of *PROX1* shifts the transcriptional profile of highly aggressive SW480R cells toward more quiescent phenotype reminiscent of SW480A cells, characterized by higher levels of cell adhesion molecules, slower growth and metabolic rates, and inability to grow in an adhesion-independent manner.

In Vivo Overexpression of PROX1 Promotes Intestinal Tumor Progression

To study the in vivo role of PROX1 in CRC, we produced transgenic mice expressing PROX1 in intestinal epithelium under the control of the 12.4 kb mouse villin promoter (Madison et al., 2002; and Figures 7A and 7B). The transgenic mice did not display obvious abnormalities of intestinal differentiation or proliferation, although the transgenic males were slightly smaller than their littermates (23.6 g versus 27.1 g, $p = 0.0092$, $n = 9$ for the tg and 10 for the WT, age 2 months). The villin-PROX1 mice did not develop intestinal tumors, at least when analyzed at the age of 1 year ($n = 6$ for WT or tg, data not shown), indicating that PROX1 overexpression alone is not sufficient to drive the oncogenic process. However, upon treatment with the colon carcinogen AOM, the villin-PROX1 mice developed significantly more numerous and larger intestinal adenomas in comparison to the wild-type littermates (Figures 7C and 7D). Notably, 9 out of 14 transgenic mice developed at least one macroscopical lesion (>1 mm^3, range 1.2–5.6 mm^3), while no large lesions were detected in any of the wild-type mice ($n = 12$, Figure 7D).

The colonic neoplasms in both wild-type and transgenic mice were adenomas, many of which extended through the lamina propria but showed no invasion of muscularis mucosae. Notably, 38% of the tumors from the villin-PROX1 transgenic mice contained high-grade dysplasia or carcinoma in situ (5/13 tumors studied) versus only 10% in the wild-type mice (1/10). We did not observe obvious differences in tumor cell proliferation or expression of neuroendocrine markers, as determined by staining of tumors of comparable size for phosphohistone H3 and chromogranin A. Interestingly, the number of preneoplastic ACFs was similar in the wild-type and transgenic mice (28.9 ± 9.0 versus 24.1 ± 6.7, $p = 0.14$, $n = 11$ and 14). Since ACFs are the putative precursors of CRC, these data support our hypothesis that PROX1 is important for tumor establishment and progression but not for tumor initiation.

Suppression of PROX1 in Colon Cancer Cells Inhibits Tumor Growth

Next we asked whether loss of PROX1 affects CRC development in vivo. We downregulated *PROX1* mRNA in using

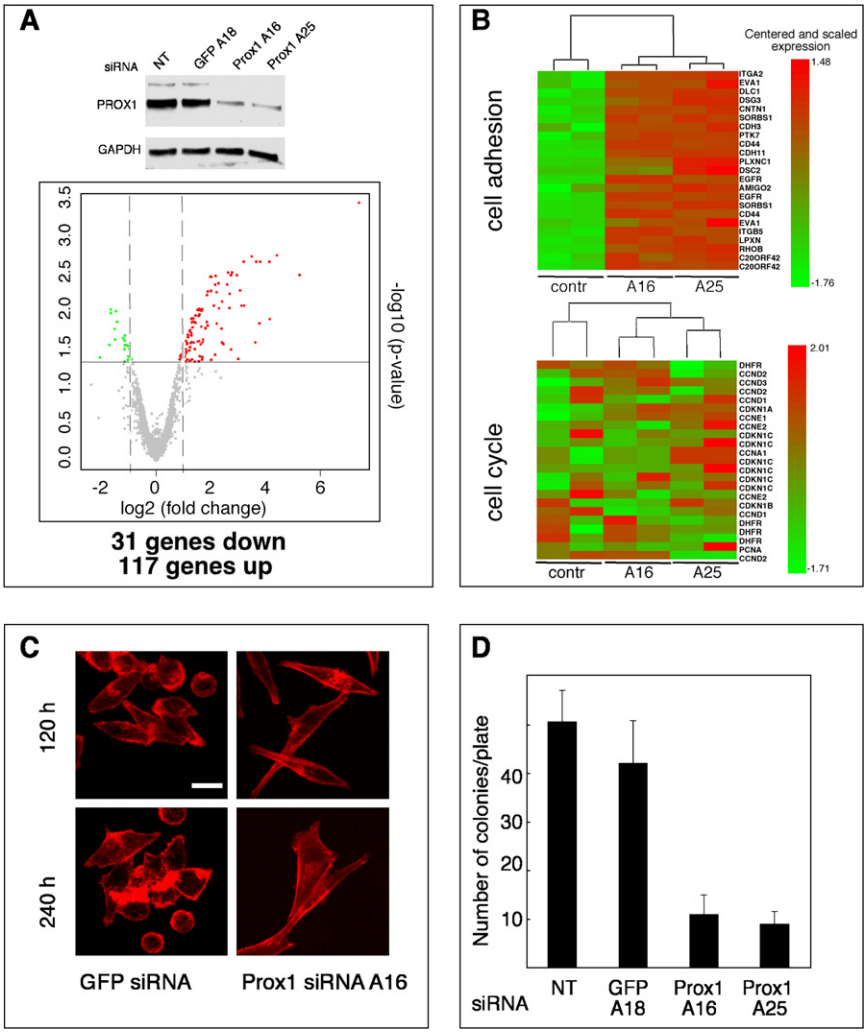

Figure 6. PROX1 Controls Cell Adhesion Program in CRC

(A) Loss of PROX1 significantly changes the expression of 148 genes. Significantly changed genes (corrected $p < 0.05$) are shown in red (upregulated) and green (downregulated) in the Volcano plot. Vertical lines mark the 2-fold change limits, and the horizontal line marks the cut-off for statistical significance.

(B) Loss of PROX1 results in the coordinate regulation of cell adhesion genes at 48 hr, while no significant changes are observed in cell-cycle-related genes. Heat maps show results of two-dimensional hierarchical clustering of samples and the 23 significantly differentially expressed probe sets targeting genes involved in cell adhesion or a selected set of cell-cycle-related genes.

(C) Loss of PROX1 induces changes in the actin cytoskeleton. Staining with falloidin 120 hr and 240 hr after PROX1 siRNA A16 or control A18 transfection.

(D) Loss of PROX1 prevents growth in soft agar. SW480R cells were transfected with GFP siRNA, PROX1si RNA A16, or PROX1 siRNA A25 or left untreated (NT) and seeded in soft agar, and the colonies were scored after 2 weeks. Data are shown as mean ± SEM.

Scale bar, 10 μm.

doxycycline-inducible *PROX1* shRNA constructs transfected to SW480R cells. Doxycycline treatment significantly reduced PROX1 expression, as well as the size and incidence of the SW480/PROX1si tumors in *nu/nu* mice, but it had no effect on SW480/GFPsi tumors (Figures 7E and 7F and Table S5). Decreased tumor growth was observed when the doxycycline treatment was initiated either immediately after implantation or 2 weeks later. Similarly, PROX1 suppression significantly inhibited growth of the PROX1-positive COLO205 CRC cells in vivo, but not in vitro (Figures 7G and 7H and data not shown).

Loss of Prox1 Inhibits Tumor Progression in $Apc^{min/+}$ Mice and Leads to Re-Establishment of Cell Polarity and Quiescence

Because of potential caveats associated with the gene silencing approach, we studied whether genetic loss of *Prox1* affects the development of intestinal polyposis in $Apc^{min/+}$ mice. We crossed mice with a conditionally targeted *Prox1* allele ($Prox1^{flox/flox}$ [Harvey et al., 2005]) with villin-Cre mice (Madison et al., 2002) and $Apc^{min/+}$ mice, confirmed an efficient deletion of *Prox1* in intestinal epithelial cells and adenomas (Figure S9), and then monitored the incidence and size of intestinal lesions in the triple transgenic mice (ΔProx1) versus the control wild-type and heterozygous littermates.

We quantified the number and size of intestinal polyps using whole-mount staining of intestinal epithelium with methylene blue, which highlights both micro- and macroscopic lesions. There was a strong reduction in the number of macroscopically visible lesions and tumor size (Figure 8A). Most notably, we observed a five-fold reduction in the number of tumors exceeding 5 mm^3 ($p = 0.02$), while the number of microscopic lesions increased (Figure 8A). Since the total number of intestinal lesions was only marginally reduced in Δ*Prox1* versus the control mice (48.7 versus 57.1, $p = 0.26$), we conclude that the loss of Prox1 does not prevent tumor initiation but instead impairs tumor progression.

The macroscopic tumors from the wild-type mice displayed a pleomorphic histology ranging from tubular adenomas of low-grade dysplasia to carcinoma in situ. The neoplastic tubules of the wild-type tumors contained enlarged, rounded nuclei, and frequent mitotic figures were readily observed in the luminal portion of the dysplastic epithelium. The dysplastic glands occurred frequently in a "back-to-back" organization and contained tumor cells with defective polarity (Figure 8B and Figure S9). In contrast, Δ*Prox1* tumors consisted of monomorphic, well-organized large glands separated by an abundant fibromuscular stroma. (Figure 8B and Figure S9). These glands contained uniformly sized, well-polarized, and tightly packed cells with elongated nuclei and infrequent mitotic figures. The rate of apoptosis did not differ between the wild-type and Δ*Prox1* tumors as

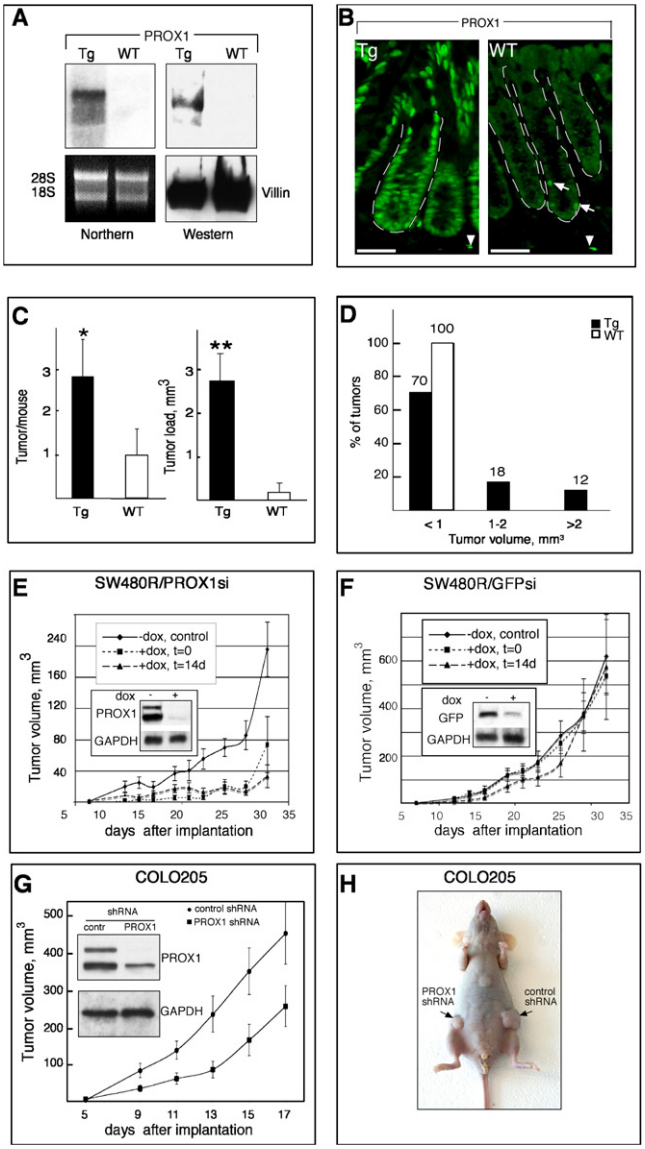

Figure 7. PROX1 Is Necessary for Tumor Growth In Vivo

(A) PROX1 expression in intestines of the villin-PROX1 transgenic and wild-type mice. Northern and western blotting for the indicated mRNA and proteins. 28S and 18S RNA and villin are used as loading controls.

(B) Expression pattern of PROX1 transgene and endogenous Prox1 in mouse colon. Arrowheads: Prox1-positive lymphatic vessels. Arrows: Prox1-positive cells in the crypt compartment.

(C) Increased tumor multiplicity and tumor load in AOM-treated villin-PROX1 transgenic mice. n = 12 and 14 for the WT and tg mice. *$p < 0.05$; **$p < 0.01$; data are shown as mean ± SEM.

(D) Tumor size distribution in villin-PROX1 transgenic and wild-type mice. Numbers above bars indicate percentage of tumors.

(E and F) Loss of PROX1 inhibits growth of SW480R tumor xenografts. Similar results were obtained with two independent clones of SW480R/PROXsi cells.

(G and H) PROX1 downregulation inhibits growth of COLO205 xenografts.

In (E)–(G), n = 10 per experimental group; data are shown as mean ± SEM. Scale bars, 25 μm.

determined by staining for active caspase 3 (data not shown). *ΔProx1* tumors, however, showed low frequency of mitotic figures and almost 70% reduction of cells stained for the proliferation marker Ki67 (Figure 8C). The nuclei of *ΔProx1* cells were darker and the nucleoli were smaller in comparison to the control tumor nuclei (Figure 8B). Since increased nucleolar size is linked to ribosome biogenesis and cell transformation (Ruggero and Pandolfi, 2003), these finding suggest a reduced protein synthesis rate in *ΔProx1* cells, which is in agreement with downregulation of protein translation pathways in PROX1 siRNA-treated cells and further confirms the transition of *Prox1* deficient cells toward a quiescent state.

Differentiation toward the goblet cell lineage via suppression of the Notch pathway blocks cell proliferation in $Apc^{min/+}$ adenomas (van Es et al., 2005). We therefore studied whether loss of Prox1 affected tumor differentiation status. Δ*Prox1* and control adenomas contained no enteroendocrine and few goblet and Paneth cells, and the expression of villin was strongly reduced in comparison to the normal mucosa (Figure S9 and data not shown). These data suggested that the loss of Prox1 does not promote cell differentiation. However, Δ*Prox1* neoplastic cells had a continuous apical staining for villin, while its localization was frequently discontinuous, patchy, or completely absent in the control adenomas (Figure 8D). Furthermore, while abundant cytoplasmic/nuclear β-catenin was present in both Δ*Prox1* and the control tumors, higher levels of β-catenin could be found in cell-cell junctions in Δ*Prox1* adenomas (Figure 8E). The better organization of Δ*Prox1* glands and upregulation of cell adhesion pathways in gene-expression analysis suggested re-establishment of cell-ECM interactions. Indeed, staining for collagen IV, fibronectin, and laminin revealed well-organized basement membranes around the Δ*Prox1* adenomas (Ø > 2 mm), which were more similar to the ones around the normal nontransformed intestinal epithelium than to the wild-type adenomas of the same size (Figure 8F, Figure S9, and data not shown). As these differences were observed in tumors of similar size, they did not result from the analysis of tumors at different stages, i.e., microscopic versus macroscopic lesions. We therefore conclude that the deletion of *Prox1* in intestinal adenomas leads to a quiescent phenotype, characterized by preservation of cell polarity, better organization of basement membranes, and strong reduction in cell proliferation, which ultimately prevents tumor growth and progression toward a more malignant phenotype.

DISCUSSION

Our study shows that the PROX1 homeobox transcription factor marks the transition from benign colon adenoma to carcinoma in situ and that its misexpression promotes tumor progression in CRC models in mice. These results demonstrate a role for this developmentally important transcription factor in cancer and show that PROX1 acts as an essential downstream effector of TCF/β-catenin signaling in CRC. Until recently, PROX1 was mostly studied in the context of embryonic development. Prox1 was shown to control the development of the lymphatic vasculature, lens fiber cells, and liver and retinal progenitor cell differentiation (Dyer et al., 2003; Sosa-Pineda et al., 2000; Wigle et al., 1999; Wigle and Oliver, 1999). One previous study has reported increased *PROX1* mRNA in colorectal tumors, but the authors attributed this to increased lymphangiogenesis (Parr and Jiang, 2003). In contrast, we show here that, in addition to lymphatic vessels, PROX1 is highly expressed in the CRC cells.

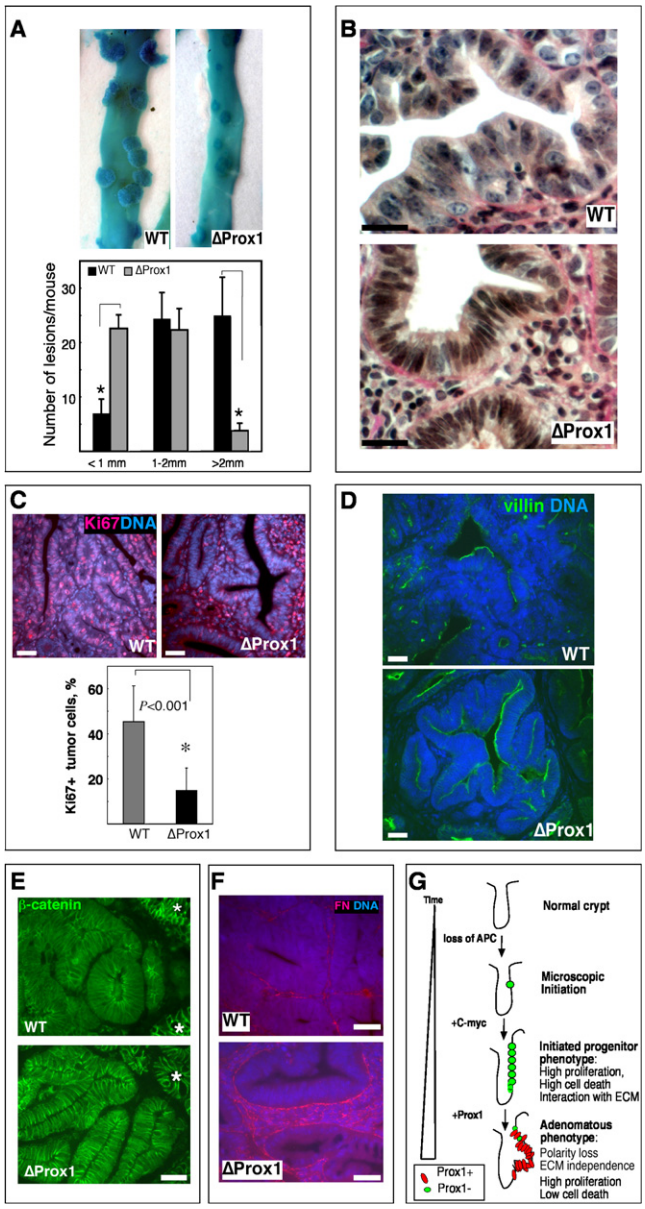

Figure 8. Intestinal-Specific Deletion of Prox1 Inhibits Tumor Progression in *Apc*$^{min/+}$ Mice

(A) Deletion of Prox1 reduces macroscopic tumor load in small intestines of *Apc*$^{min/+}$ mice (n = 8, WT, and n = 11, ΔProx1, age 24 weeks).

(B) Altered cell morphology in macroscopic ΔProx1 tumors. Note preservation of cell polarity and elongated nuclei in ΔProx1 versus the control tumors.

(C) Reduced cell proliferation in ΔProx1 versus the WT tumors. Five tumors (Ø = 2 mm) from three animals of each genotype (15 total/genotype) were used to calculate Ki67 proliferation index.

(D–F) (D) Preservation of apical-basal polarity in macroscopic ΔProx1 tumors (Ø > 2 mm). Staining for apical marker villin (green) and DNA (blue). (E) Increased β-catenin in cell-cell junctions of ΔProx1 tumors. Note similar levels of β-catenin in normal glands of the wild-type and ΔProx1 tumors (asterisks). (F) Better preservation of basement membrane in ΔProx1 tumors. Staining for fibronectin (red) and DNA (blue). For (D)–(F), five to ten tumors of similar size per genotype were examined.

(G) A model for PROX1 role in CRC. Following loss of *Apc* and activation of TCF/β-catenin signaling, intestinal epithelial cells begin to proliferate and express genes associated with the intestinal progenitor cell program. C-myc is a direct target of TCF/β-catenin that controls most of downstream effects of this pathway. Prolonged and high TCF/β-catenin activation, possibly in cooperation with other events, induces Prox1 in the initiated progenitor cells, which leads to the suppression of cell dependency on cell-cell and cell-ECM contacts, loss of cell polarity and tissue architecture, further uncontrolled proliferation, and establishment of macroadenomas and carcinomas in situ.

Data in (A) and (C) are shown as mean ± SEM. Scale bars, 25 μm.

Our data indicate a gain of function via PROX1 expression, which contrasts with the reported tumor suppressor role for Prospero in *Drosophila* (Betschinger et al., 2006). Asymmetric distribution of Prospero during larval neuroblast division inhibits the self-renewal of one of the daughter cells and maintains a balance between the neuroblast stem-like cell population and ganglion mother cells that give rise to neurons. Loss of Prospero shifts the balance toward uncontrolled proliferation through loss of control of cell-cycle genes, such as dcycE and leads to formation of tumors with neuroblastoma-like features (Betschinger et al., 2006; Caussinus and Gonzalez, 2005). In mice, Prox1 controls cell-cycle exit of mouse retinal progenitor cells and promotes horizontal-cell fate, loss of Prox1 increases the proliferation of lens fiber cells, and Prox1 also participates in the production of "secondary transition" pancreatic endocrine cells (Dyer et al., 2003; Wang et al., 2005; Wigle et al., 1999). We found that Prox1 is expressed in the Ngn3+ progenitor cells as well as in a subset of mature enteroendocrine cells in the intestinal epithelium. Its function in these cells is likely to be evolutionarily conserved, as Prospero expression identifies enteroendocrine cells in *Drosophila* midgut (Ohlstein and Spradling, 2007). In mammals, enteroendocrine cells are composed of at least 15 different subpopulations. Previous work suggests the existence of two pathways for enteroendocrine differentiation, one producing serotonin and substance P, and another expressing PYY, CCK, neurotensin, and GLP-1 (Roth et al., 1992). Prox1 is preferentially expressed in the latter cells, and we speculate that one of the functions of Prox1 in the normal intestinal epithelium is the regulation of enteroendocrine progenitor commitment to one of the two pathways.

In contrast to *Drosophila* ganglion mother cells, vertebrate retinal progenitors, or gut enteroendocrine cells, we find that Prox1 is highly expressed in cultured, actively proliferating CRC cells and in intestinal adenomas. Moreover, downregulation or deletion of *PROX1* inhibits tumor progression, while PROX1 overexpression potentiates colonic adenoma development. These results demonstrate that, in the context of oncogenic signaling, PROX1 contributes to tumor progression. We suggest that, in colon cancer, Prox1 activity is integrated into genetic circuits other than regulation of the cell cycle and terminal differentiation, and that in dysplastic intestinal epithelial cells this function manifests as abnormal control of cell adhesion, ECM interactions, and cell polarity. This conclusion is based on several observations. First, Prox1-positive cells in CRC do not express neuroendocrine markers that are coexpressed with Prox1 in normal cells, and loss of Prox1 does not lead to differentiation toward any specific cell lineage. Furthermore, PROX1 overexpression in PROX1-negative CRC cells does not promote cell-cycle exit or neuroendocrine phenotype (T.V.P., unpublished data). Second, PROX1 suppression in vitro does not directly affect cell-cycle regulators, but instead induces genes associated with cell

adhesion pathways, and actin cytoskeleton remodeling and leads to the cell reprogramming toward an adhesion-dependent and quiescent phenotype. Finally, decreased tumor cell proliferation alone upon deletion of cell-cycle regulator *cyclin D1* in $Apc^{min/+}$ mice leads to a different phenotype, characterized by a lack of changes in tumor cell morphology and a reduction in tumors at all stages (Wilding et al., 2002). Therefore, we suggest that Prox1 contributes to tumor progression by disrupting tissue architecture, cell polarity, and adhesion, which in the context of oncogenic Wnt signaling leads to spatially unrestricted cell proliferation. Our preliminary comparison of gene expression profiles following loss of β-catenin versus loss of Prox1 in CRC cells shows that Prox1 controls a functionally distinct subset of β-catenin-responsive genes (unpublished data).

What controls the expression of Prox1 in tumors? We propose that, during the genesis of colon cancer, PROX1 expression is triggered in response to abnormally high and prolonged TCF/β-catenin signaling. In these conditions, PROX1 acts as a switch converting the original "normal progenitor" TCF/β-catenin program, in which the progenitor cells are still responsive to extracellular signals from underlying basement membrane and are relatively well polarized, to the "adenomatous progenitor" TCF/β-catenin program, in which cells are able to survive and proliferate under unfavorable conditions, despite cell crowding, growth inhibitory signals from stromal cells, decreased ECM adhesion, and loss of cell polarity (Figure 8G). This would increase retention of adenomatous cells in the tissue, further predisposing them to the accumulation of harmful genetic changes, such as *KRAS* and *P53* mutations that lead to the development of full-blown colon cancer. This hypothesis is consistent with the expression of Prox1 in adenomatous cells, but not in expanded progenitors upon *Apc* deletion in vivo, high PROX1 levels in human high-grade dysplastic lesions, inability of cancer cells to form colonies in soft agar or tumors in vivo upon loss of Prox1, and potentiation of tumor growth and progression, but not tumor initiation in villin-PROX1 transgenic mice.

A remaining outstanding question is the exact mechanism of PROX1 action in colon cancer. The molecular effectors of this transition remain to be elucidated. PROX1 modifies the expression of at least 150 genes in vitro, and we believe that the observed effect of PROX1 on the development of colon tumors is likely to be due to changes in the transcription of many genes, rather than of any single ones. Thus, further studies, such as identification of the direct PROX1 target genes in CRC, are necessary to elucidate the molecular details of PROX1 function in this disease. Knowledge of direct Prox1 targets in diverse mammalian cell types and comparison with targets of Prospero in *Drosophila* should provide the basis for a detailed understanding of the evolutionarily conserved, as well as divergent, pathways controlled by this transcription factor in normal and cancer cells.

One common theme regarding the function of Prox1 is the importance of the cellular context. Indeed, a comparison of the genes regulated by PROX1 in lymphatic endothelial, colon, and liver cancer cells shows very little overlap, demonstrating that PROX1 controls highly specific transcriptional programs in each context (our unpublished data). The control of PROX1 expression is itself highly tissue specific, as β-catenin/TCF signaling strongly induces Prox1 in the intestinal epithelium but has little effect in hepatocytes. These results demonstrate important tissue-specific differences in signaling downstream of the activated Wnt pathway in cancers of the intestine and liver. Such cell type-specific responses are likely to be important determinants of tumor progression in these organs. We identified a conserved 350 bp element 49 kb upstream of the *PROX1* transcription start site, which contains TCF/LEF binding sites that confer the responsiveness to Wnt signaling in vitro. This region also contains conserved binding sites for RORα, SOX17, MZF1, Snail, SPI-B, and CSL (Figure S6). Future studies, such as generation of mice with targeted deletions of these transcription factor binding sites, should identify pathways that are responsible for tissue-specific induction of Prox1 in response to Wnt signaling. Prox1 expression is also lost upon inactivation of Wnt signaling in cerebellar granule cells; this could be another tissue where the TCF/LEF-responsive enhancer participates in Prox1 transcription (Zhou et al., 2004).

In conclusion, our results show that PROX1 does not act as a tumor suppressor in colon cancer; conversely, its expression is activated in response to abnormally elevated oncogenic TCF/β-catenin signaling in intestinal epithelium, and this overexpression is important for tumor progression via disruption of cell polarity and adhesion. Our data indicate that PROX1 is not required for the early stages of tumor development, such as the microscopic initiation and expansion of preneoplastic progenitor cells, which produce clinically benign lesions. Instead, PROX1 is important for the establishment and growth of highly dysplastic in situ lesions, which, if left untreated, will inevitably progress to cancer. We believe that these results reflect the multistage, tissue-specific nature of the carcinogenesis process. Our results also reinforce the concept that, during tumor progression, cancer cells can misconnect and integrate activities from multiple pathways, including those linked to the differentiation of the corresponding normal cell types. Although an abnormally activated Wnt pathway is the driving force in intestinal carcinogenesis, its complete inhibition will likely cause significant side effects in cancer patients because Wnt signaling plays an important role in normal intestinal tissue homeostasis (Pinto et al., 2003). Thus, silencing the activity of colon cancer-specific targets of the Wnt pathway, such as PROX1, may represent another strategy for fighting this difficult disease.

EXPERIMENTAL PROCEDURES

Reagents

Antibody suppliers, details of immunohistochemical procedures, and synthetic RNAs (Dharmacon Research) are provided in the Supplemental Experimental Procedures.

Cell Culture, Transfection, ChIP, Soft Agar Assay, and EMSA Analysis

SW480, HT29, SW48, DLD1, WiDr, COLO205, HCT-116, and LS174T cells were cultured under conditions suggested by the provider (ATCC). Subclones of SW480 cells were derived by the limiting dilution method. Production of stable cell lines for tumor xenograft experiments is described in the Supplemental Experimental Procedures. ChIP was performed as described (O'Geen et al., 2006). For soft agar assay, 2×10^3 cells were seeded in triplicate in 1 ml of 0.33% (w/v) agar (Difco) in complete D-MEM medium in 6-well plates on 1 ml of a 0.5% bottom agar layer. Cells were fed twice a week, and the number of colonies per well was scored 2 weeks later. EMSA was carried out using 5′-biotinylated DNA oligonucleotides containing a 9 bp TCF/LEF binding sequence flanked by 10 bp of the surrounding promoter sequences; in control

oligonucleotides the core binding sequence was scrambled (see Supplemental Experimental Procedures).

RNA Isolation, Northern and Western Blotting, and Gene Expression Profiling

Total RNA was isolated using RNeasy columns (QIAGEN). The Cancer Profiling Array (Clontech) was hybridized with ^{32}P-labeled probes for LYVE-1 (AF118108) and PROX1 (NM002763), and the signals were normalized to that of GAPDH. Northern blots were hybridized with ^{32}P-labeled probes (see Supplemental Experimental Procedures), and western blots were developed using the ECL method.

For the analysis of differential gene expression, RNA was isolated from two independent clones of SW480R or SW480A cells. For the analysis after PROX1 knockdown, SW480R cells were transfected with PROX1 siRNA A16 or A25 or GFP siRNA A18; RNA was isolated 48 hr later and processed for array hybridization, data validation, and pathway analysis (see Supplemental Experimental Procedures). Microarray data were submitted to MIAMExpress at the European Bioinformatics Institute, Hinxton, UK (http://www.ebi.ac.uk/arrayexpress/) under the experimental accession number E-MEXP-1422.

In Vivo Experiments

Experiments were approved by the Helsinki University Ethics Committee. Genotyping was as described (Harvey et al., 2005; Sterner-Kock et al., 2002; Su et al., 1992). *Apc*$^{min/+}$ and villin-Cre mice (Jackson laboratories) were on the C57BL6J background, and *Prox1*$^{flox/flox}$ mice were bred for five generation to the C57BL6J background. For tumor xenografts, *nu/nu* NMRI male mice were injected subcutaneously with 1 × 10^6 SW480R/si cells or 2 × 10^6 COLO205/si cells, and tumors were allowed to grow for up to 5 weeks. Doxycycline (1 mg/ml) in 0.5% sucrose was administered in drinking water after injection or 2 weeks later; the control group received 0.5% sucrose. Tumor size was calculated as length × width^2 × 0.52. Villin-PROX1 transgenic mice on FVB/N background expressed PROX1 cDNA under the 12.4 kb mouse villin promoter. Mice were genotyped by PCR using the following primers: 5′-CCGGGTGGGCAGGGTAGAGG, 5′-ACAGCTGGGAAATTATGGTTGC.

AOM Treatment and Intestinal Tumor Analysis

Six-month-old transgenic and littermate wild-type mice received five weekly injections of 10 mg/kg of AOM (Sigma) and were sacrificed 10 weeks after the last injection. Large (AOM treatment) and small (*Apc*$^{min/+}$ analysis) intestines were slit open, mounted flat, fixed in 4% PFA, stained with 0.1% methylene blue, and analyzed using published guidelines (Boivin et al., 2003) by two observers (T.V.P. and A.N.). Histological scoring was without knowledge of the genotype by an experienced pathologist (L.C.A.).

Statistical Analysis

A two-tailed, unpaired Student's t test was done to determine statistical significance by the probability of difference between the means. $p < 0.05$ is considered statistically significant. Values in the graphs are expressed as means ± SEM or SD.

ACCESSION NUMBERS

Microarray data were submitted to MIAMExpress at the European Bioinformatics Institute, Hinxton, UK (http://www.ebi.ac.uk/arrayexpress/) under the experimental accession number E-MEXP-1422.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, nine supplemental figures, and five supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/13/5/407/DC1/.

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