stimuli, leading to uncontrolled transcription of the *hTcf-4* target genes. The apparent de novo expression of other members of the Tcf family in some colon carcinoma cell lines might lead to a further deregulation of *Tcf* target gene expression by the same mechanism. The control of β-catenin–Tcf signaling is likely to be an important part of the gatekeeper function of APC (*19*), and its disruption may be an early step in malignant transformation.

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11. A genomic fragment encoding the HMG-box region of hTcf-4 (*7*) was used to probe a human 12-week fetal cDNA library in Lambda GT-11. Positive clones were subcloned into pBluescriptSK and sequenced.
12. Northern blot hybridizations (*7*) were performed with full-length *hTcf-1*, *hLef-1*, and *hTcf-4* cDNA. Colon epithelial cells were freshly prepared from a mucosal preparation dissected from a healthy surgical colon sample. The sample was minced and incubated with 1 mM dithiothreitol (DTT) in Hanks' medium to remove mucus. Single-cell suspensions were prepared by incubation at room temperature in 0.75 mM EDTA in Hanks' medium. Epithelial cells were separated from lymphocytes by Percoll gradient centrifugation.
13. In situ hybridization of 6 μm frozen sections of healthy colon biopsy samples was performed as described [E. van Hoffen *et al.*, *Am. J. Pathol.* **149**, 1991 (1996)]. *hTcf-4* cDNA encoding amino acids 200 to 310 was amplified and labeled with Dig-11-dUTP (Boehringer Mannheim) by PCR. After hybridization and washing, the sections were sequentially incubated with mouse antibody to Dig (Boehringer) and a horseradish peroxidase–conjugated rabbit antibody to mouse immunoglobulin (Dako, Glostrup, Denmark). The signal was visualized with diaminobenzidine, which produces a reddish-brown precipitate. Blue counterstaining was performed with hematoxylin.
14. Reporter gene assays were performed as in (*7*). In brief, 2×10^6 cells were transfected with plasmids by electroporation. After 24 hours, cells were harvested and lysed in 1 mM DTT, 1% Triton X-100, 15% glycerol, 25 mM tris (pH 7.8), and 8 mM $MgCl_2$. Complementary DNAs encoding Myc-tagged versions of β-catenin and hTcf-4 were inserted into the mammalian expression vector pCDNA (Invitrogen). Sequences of pTOPCAT, pFOPCAT, pTOPFLASH, and pFOPFLASH are available upon request. pCAT-CONTROL, encoding the CAT enzyme under the control of the SV40 promoter, was purchased from Promega.
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18. Gel retardation assays were performed as described (*7*). Extracts were prepared from intact nuclei that were washed four times to avoid contamination with cytoplasmic β-catenin. As the optimal Tcf-Lef probe, we used a double-stranded 15-nucleotide oligomer CCCTTTGATCTTACC; the control probe was CCCTTTGGCCTTACC. All oligonucleotides were from Isogen (Maarssen, Netherlands). The β-catenin antibody was purchased from Transduction Laboratories (Lexington, KY). A typical binding reaction contained 3 μg of nuclear protein, 0.1 ng of radiolabeled probe, and 100 ng of deoxyinosine-deoxycytidine (dIdC) in 25 μl of binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol). Samples were incubated for 20 min at room temperature, antibody was added, and the samples were incubated for a further 20 min.
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Activation of β-Catenin–Tcf Signaling in Colon Cancer by Mutations in β-Catenin or APC

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Inactivation of the adenomatous polyposis coli (*APC*) tumor suppressor gene initiates colorectal neoplasia. One of the biochemical activities associated with the APC protein is down-regulation of transcriptional activation mediated by β-catenin and T cell transcription factor 4 (Tcf-4). The protein products of mutant *APC* genes present in colorectal tumors were found to be defective in this activity. Furthermore, colorectal tumors with intact *APC* genes were found to contain activating mutations of β-catenin that altered functionally significant phosphorylation sites. These results indicate that regulation of β-catenin is critical to APC's tumor suppressive effect and that this regulation can be circumvented by mutations in either APC or β-catenin.

Mutations of the *APC* gene are the most common disease-causing genetic events in humans; about 50% of the population will develop colorectal polyps initiated by such mutations during a normal life-span (*1*). Individuals who inherit *APC* mutations develop thousands of colorectal tumors, consistent with the tumor suppressor or "gatekeeping" role of APC protein in colorectal tumorigenesis (*2*, *3*). APC homodimerizes through its NH_2-terminus (*4*) and interacts with at least six other proteins: β-catenin (*5*), γ-catenin (plakoglobin) (*6*), tubulin (*7*), EB1 (*8*), hDLG, a homolog of the *Drosophila* Discs Large tumor suppressor protein (9), and glycogen synthase kinase–3β (GSK-3β) (*10*), a mammalian homolog of ZW3 kinase. Whether any of these interacting proteins communicate APC growth-controlling signals is unknown. Here, we used a genetic approach to investigate the role of β-catenin in APC's tumor suppressor function.

Although β-catenin was originally discovered as a cadherin-binding protein, it has recently been shown to function as a transcriptional activator when complexed with members of the Tcf family of DNA binding proteins (*11*). One family member, hTcf-4, is expressed in normal and neoplastic colorectal epithelium, and wild-type (WT) APC can suppress signaling by the β-catenin–Tcf complex (*12*). If this inhibitory activity is critical for APC's tumor suppressor function, then mutant APC proteins should be defective in this activity.

To evaluate this hypothesis, we tested four *APC* mutants (Fig. 1A) for their ability to inhibit β-catenin–Tcf–regulated transcription (CRT) in transfection assays. The first mutant, APC331Δ, represents a type of mutation commonly found in the germ line of familial adenomatous polyposis patients as well as in sporadic tumors (*2*). The APC331Δ protein is truncated at codon 331, NH_2-terminal to the three 15–amino acid (aa) β-catenin–binding repeats between codons 1020 and 1169. The second mutant, APC1309Δ, is the most common germline *APC* mutation (*2*), a 5–base pair (bp) deletion that produces a frameshift at

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codon 1309 and truncation of the protein. The APC1309Δ protein retains the 15-aa β-catenin–binding repeats but lacks the seven 20-aa repeats between codons 1323 and 2075 that have been implicated in binding and degradation of β-catenin (*5*). The third mutant, APC1941Δ, represents one of the most distal somatic mutations observed in colorectal tumors (*13*). The APC1941Δ protein is truncated at codon 1941 and therefore contains the 15-aa repeats and all but the last two 20-aa repeats. Finally, APC2644Δ represents a germline mutation resulting from a 4-bp deletion in codon 2644. Patients with this type of unusual COOH-terminal mutation develop few polyps (attenuated polyposis) but have pronounced extracolonic disease, particularly desmoid tumors (*14*).

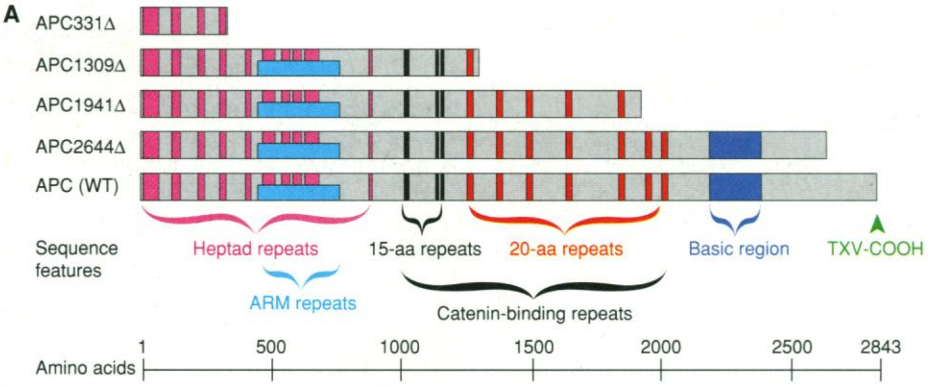
Each of the *APC* mutants was cotransfected with a CRT reporter into the SW480 colorectal cancer cell line. SW480 cells have truncated APC and constitutively active CRT, which can be suppressed by exogenous WT APC (*12*). Although all four mutants produced comparable amounts of APC protein after transfection (*15*), they varied in their CRT inhibitory activity. The three mutants found in patients with typical polyposis or cancer were markedly deficient in inhibition of CRT (Fig. 1B). The reduced activity of APC1309Δ and APC1941Δ suggests that β-catenin binding is not sufficient for APC-mediated inhibition of CRT and that the complete set of 20-aa repeats is required. The inhibitory activity of the APC2644Δ mutant associated with attenuated polyposis was comparable with that of WT APC (Fig. 1B), suggesting that the DLG-binding domain at the COOH-terminus of APC is not required for down-regulation of CRT.

If APC's inhibition of CRT is critical to suppression of colorectal tumorigenesis, cancers with WT APC must escape this inhibition through alternative mechanisms. To investigate this possibility, we evaluated CRT in two colorectal tumor cell lines (HCT116 and SW48) that express full-length APC (Fig. 2A). Both HCT116 and SW48 displayed constitutively active CRT and, in contrast to cell lines with truncated APC (DLD1 and SW480), this activity was not inhibited by exogenous WT APC (Figs. 1B and 2B). Other (noncolorectal cancer) cell lines expressing WT APC do not display constitutive CRT activity (*12*).

These transfection results suggested that the constitutive CRT in HCT116 and SW48 might be due to an altered downstream component of the APC tumor suppressor pathway. We therefore evaluated the status of a likely candidate, β-catenin, in the same four lines. All four lines expressed similar amounts of apparently intact β-catenin, as assessed by immunoblots (Fig. 3A). However, sequence analysis revealed that both HCT116 and SW48 harbored mutations in the β-catenin gene (*CTNNB1*) (Fig. 3B). HCT116 had a 3-bp deletion that removed one amino acid (Ser^{45}) and SW48 had a C-to-A missense mutation that changed Ser^{33} to Tyr. Analysis of paraffin-embedded archival tissue from the HCT116 patient confirmed the somatic nature of this mutation and its presence in the primary tumor before culture (*16*). Both mutations affect serines that have been implicated in the down-regulation of β-catenin through phosphorylation by the GSK-3β kinase in *Xenopus* embryos (Fig. 3C) (*17*, *18*).

To test the generality of this mutational mechanism, we evaluated five primary colorectal cancers in which sequencing of the entire coding region of *APC* had revealed no mutations (*13*). Three of these five tumors were found to contain *CTNNB1* mutations (S45F, S45F, and T44A) that altered potential GSK-3β phosphorylation sites (*19*) (Fig. 3C). Each mutation was somatic and appeared to affect only one of the two *CTNNB1* alleles.

Because the β-catenin mutations were heterozygous, we hypothesized that the mutations might exert a dominant effect, rendering a fraction of cellular β-catenin insensitive to APC-mediated down-regulation. To test this notion, we performed gel shift analyses with nuclear extracts from untransfected HCT116 cells. In contrast to noncolorectal cancer cell lines with intact *APC*, HCT116 cells contained a β-cate-



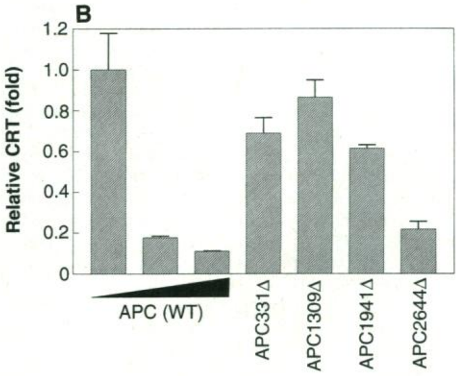
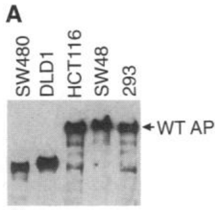


Fig. 1. Effects of *APC* mutations on CRT. (**A**) Schematic of wild-type (WT) and mutant APC. APC is a 2843-aa protein (*23*) that contains Armadillo (ARM) repeats in the NH$_2$-terminus (*24*), 15- and 20-aa β-catenin–binding repeats in the central region (*5*, *6*), and a basic region in the COOH-terminus (*23*). The COOH-terminus also contains a TXV sequence, which mediates DLG binding (*9*). (**B**) Effects of WT and mutant APC on CRT. SW480 cells containing endogenous mutant *APC* were transfected with the APC expression vectors shown in (A) and CRT was measured (*25*). Cells were transfected with increasing amounts of WT *APC* (0, 0.15, and 0.5 μg, indicated by the black wedge) or 0.5 μg of mutant *APC*. CRT reporter activities are expressed relative to assays containing no WT APC and are the means of three replicates. Error bars represent standard deviations.



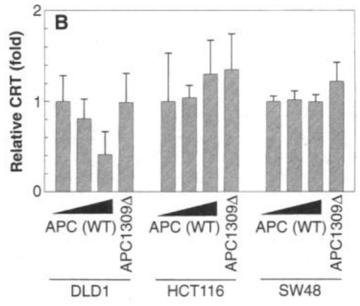


Fig. 2. Evaluation of CRT in colorectal cancer cell lines with WT APC. (**A**) Immunoblot of endogenous APC in the SW480, DLD1, HCT116, SW48, and 293 cell lines, developed with APC monoclonal antibody FE9 (*26*). (**B**) Effects of exogenous WT APC on CRT in cell lines with endogenous mutated or WT APC. Cells were transfected with increasing amounts (0, 0.15, and 0.5 μg for DLD1 and SW48; 0, 0.5, and 5 μg for HCT116) of WT APC or APC1309Δ mutant (0.5 μg for DLD1 and SW48; 5 μg for HCT116), and CRT was assessed as in Fig. 1. CRT reporter activities are expressed relative to activity in extracts without exogenous APC and are the means of three replicates. Error bars represent standard deviations.

nin–Tcf complex that gel-shifted an optimized Tcf-binding oligonucleotide, and this complex supershifted with antibody to β-catenin (Fig. 4A). We also constructed β-catenin expression vectors and compared the biologic activity of the mutant β-catenin from HCT116 (β-Cat Δ45) and SW48 (β-Cat S33Y) with that of their WT counterpart. For these experiments, we used the 293 kidney epithelial cell line because it is highly transfectable, exhibits low endogenous CRT, and contains a large amount of endogenous APC (Fig. 2A). In the presence of endogenous APC, both mutant β-catenins were at least six times as active as the WT protein, and this activity was inhibited by dominant-negative hTcf-4 (Fig. 4B).

Together, these results indicate that disruption of APC-mediated regulation of CRT is critical for colorectal tumorigenesis. This is most commonly achieved by recessive inactivating mutations of both *APC* alleles but, as shown here, can also be achieved by dominant mutations of *CTNNB1* that render CRT insensitive to the effects of WT APC. Our results suggest that APC inhibition of CRT requires phosphorylation of β-catenin at multiple sites. These potential phosphorylation sites are consistent with the known specificity of GSK-3β (*20*), a serine/threonine kinase that negatively regulates β-catenin in *Xenopus* and *Drosophila* cells (*17*) and that interacts with APC and β-catenin in mammalian cells (*10*). Our results also suggest a functional basis for the occasional *CTNNB1* mutations observed in other tumor types (*21*) and illustrate how a critical pathway in human disease can be illuminated by the discovery of mutations in different components of the pathway. The next step in understanding *APC* function will be the identification of the genes that are activated by hTcf-4–β-catenin complexes and inhibited by WT APC. These genes are likely to be related to APC's ability to induce apoptosis in colorectal cancer cells (*22*).

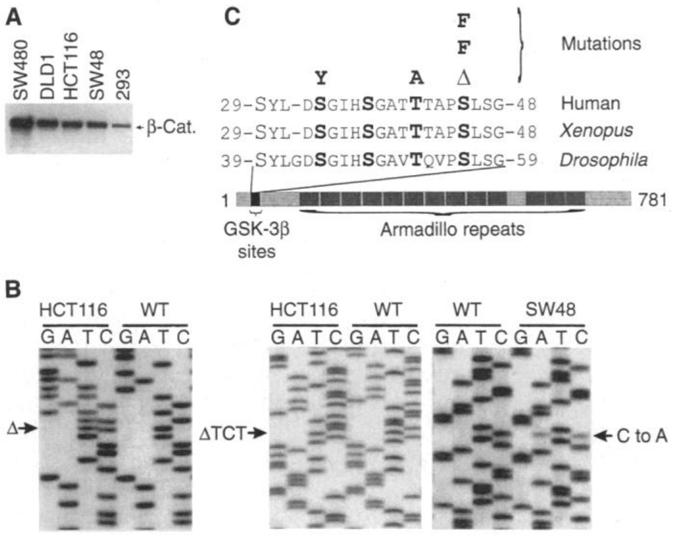


Fig. 3. Evaluation of β-catenin in colorectal cancer cell lines with WT APC. (**A**) Immunoblot of the cell lines used in this study, developed with β-catenin monoclonal antibody C19220 (Transduction Laboratories, Lexington, Kentucky) (*22*). (**B**) Sequence of *CTNNB1* in HCT116 and SW48 (*27*). The left panel (nucleotides 121 to 143 from HCT116) reveals the presence of a deletion in addition to the WT sequence. The middle panel (antisense strand of nucleotides 156 to 113 of the WT and deleted alleles of HCT116) reveals the 3-bp deletion (ΔTCT) that removed codon 45 in half the clones. The right panel (nucleotides 80 to 113 from SW48) reveals a C-to-A transition affecting codon 33 (TCT to TAT). (**C**) Schematic of β-catenin illustrating the Armadillo repeats (*24*) and negative regulatory domain. The residues in larger type fit the consensus sequence for GSK-3β phosphorylation (*20*) and those in bold have been demonstrated to affect down-regulation of β-catenin through GSK-3β phosphorylation in *Xenopus* embryos (*17*). The five mutations found in human colon cancers are indicated at the top. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

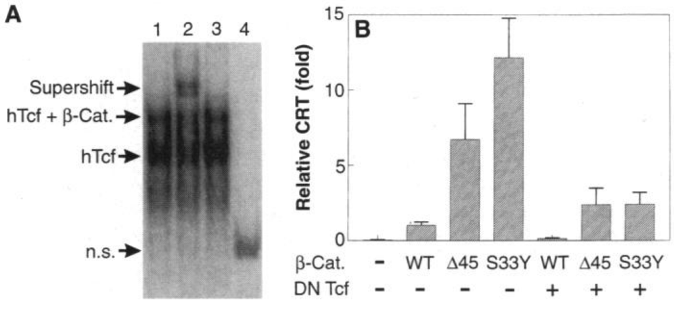


Fig. 4. Functional evaluation of β-catenin mutants. (**A**) Constitutive nuclear complex of β-catenin and Tcf in HCT116 cells. The presence of nuclear β-catenin–Tcf complexes was assessed by gel shift assays (*12*). Lanes 1 to 3, the optimal Tcf retardation probe shifted with nuclear extract from HCT116 cells when no antibody was added (lane 1) or with addition of antibody to β-catenin (0.25 μg, lane 2) or an irrelevant antibody (0.25 μg, lane 3); lane 4, mutant Tcf retardation probe shifted with nuclear extract from HCT116 cells; n.s., nonspecific shifting seen with the mutant probe. (**B**) Effects of the β-catenin mutations on CRT. Human 293 cells were transfected with WT or mutant (Δ45, S33Y) β-catenin and CRT was assessed (*28*). CRT reporter activities are expressed relative to WT β-catenin and are the means of three replicates. Error bars represent standard deviations. DN Tcf refers to dominant-negative hTcf-4.

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15. WT and mutant *APC* constructs (2 μg) were transfected into 293, SW480, and HCT116 cells with lipofectamine (Gibco-BRL, Gaithersburg, MD). Protein was harvested 24 hours later and subjected to immunoblot analysis with APC monoclonal antibody FE9 (*23*). In HCT116 and 293 cells, exogenous WT APC comigrated with the endogenous APC. In SW480 cells, APC1309Δ comigrated with the endogenous mutant APC. In all other cases, the nonfunctional APC constructs (APC331Δ, APC1309Δ, and APC1941Δ) produced as much or more protein than the CRT-functional forms of APC (WT APC and APC2644Δ).
16. Genomic DNA was isolated from paraffin-embedded normal and tumor tissue from the patient from whom the HCT116 cell line was derived [S. E. Goelz, S. R. Hamilton, B. Vogelstein, *Biochem. Biophys. Res. Commun.* **130**, 118 (1985)]. A 95-bp polymerase chain reaction (PCR) product encompassing the mutation was then amplified by PCR and directly sequenced with ThermoSequenase (Amersham). The 3-bp deletion was observed in tumor but not in normal tissue.
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19. Genomic DNA was isolated from frozen-sectioned colorectal cancers (*13*), and a 1001-bp PCR product containing exon 3 of *CTNNB1* was then amplified by PCR and directly sequenced with ThermoSequenase (Amersham). An ACC to GCC change at codon

41 (T41A) and a TCT to TTT at codon 45 (S45F) was observed in one and two tumors, respectively.

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25. Lipofectamine was used to cotransfect SW480 cells with an internal control (0.5 μg of pCMV-βgal), a reporter construct (0.5 μg of pTOPFLASH or pFOPFLASH), and the indicated amount of the various APC expression vectors. The pTOPFLASH reporter contained an optimized Tcf-binding site 5′ of a luciferase reporter gene, whereas pFOPFLASH contained a mutated site that does not bind Tcf (*12*). The amount of DNA in each transfection was kept constant by the addition of an appropriate amount of empty expression vector (pCEP4). Luciferase and β-galactosidase activities were determined 16 hours after transfection. Luciferase activity was corrected for transfection efficiency (by using the control β-galactosidase activity) and nonspecific transcription (by using the pFOPFLASH control).
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27. Overlapping segments constituting the entire *CTNNB1* were amplified by reverse transcriptase (RT)–PCR from SW480, DLD1, HCT116, and SW48 cells and sequenced directly with ThermoSequenase (Amersham). In the case of HCT116, a PCR product containing the deleted region was also cloned into pCI-neo (Promega, Madison, WI), and multiple clones corresponding to each allele were individually sequenced. Sequences of the PCR and sequencing primers used are available on request.
28. β-Catenin expression constructs were prepared as follows. WT *CTNNB1* was amplified by RT-PCR from SW480 cells and cloned into the mammalian expression vector pCI-neo (Promega) to produce pCI-neo-β-cat. The pCI-neo-β-cat Δ45 and S33Y mutants were generated by replacing codons 1 to 89 in pCI-neo-β-cat with a PCR product encoding the equivalent region from HCT116 or SW48 cDNA, respectively. The structures of all constructs were verified by sequence analysis. Details concerning the constructs and the primer sequences are available on request. Lipofectamine was used to cotransfect 293 cells with an internal control (0.1 μg of CMV-βgal), a reporter (0.5 μg of pTOPFLASH or pFOPFLASH), a Tcf-4 expression vector (0.5 μg of pCDNA-TCF4), and β-catenin (0.5 μg) or dominant-negative hTcf-4 (1.0 μg) (*12*) expression vectors. CRT was determined as in (*25*).

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Stabilization of β-Catenin by Genetic Defects in Melanoma Cell Lines

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Signal transduction by β-catenin involves its posttranslational stabilization and downstream coupling to the Lef and Tcf transcription factors. Abnormally high amounts of β-catenin were detected in 7 of 26 human melanoma cell lines. Unusual messenger RNA splicing and missense mutations in the β-catenin gene (*CTNNB1*) that result in stabilization of the protein were identified in six of the lines, and the adenomatous polyposis coli tumor suppressor protein (APC) was altered or missing in two others. In the APC-deficient cells, ectopic expression of wild-type APC eliminated the excess β-catenin. Cells with stabilized β-catenin contained a constitutive β-catenin–Lef-1 complex. Thus, genetic defects that result in up-regulation of β-catenin may play a role in melanoma progression.

The protein β-catenin is an important signaling protein in both *Xenopus laevis* and *Drosophila melanogaster* development (*1*). The proposed pathway, which is initiated by the wnt-1/wingless receptors, involves the posttranslational stabilization of β-catenin, leading to its accumulation in the cytoplasm and nucleus. In the nucleus, β-catenin is thought to interact with the Lef and Tcf families of transcription factors and thus directly regulates expression of target genes (*2*). The *wnt-1* proto-oncogene also stabilizes β-catenin in mammalian cell culture and promotes tumor formation when expressed in mouse mammary tissue (*3*). The potential role of β-catenin signaling in cancer is supported by the observation that the APC tumor suppressor protein down-regulates excess intracellular β-catenin when it is ectopically expressed in colon cancer cells containing defective APC (*4*). The regulatory mechanism for β-catenin turnover requires the NH$_2$-terminal region of the protein. Deletion of this sequence, or mutation of four serine or threonine residues therein, result in the accumulation of β-catenin and thus activate its role in signaling (*5–7*). Conceivably then, mutations that stabilize β-catenin may contribute to loss of cell growth control in tumorigenesis.

Previously, a mutant form of β-catenin, containing a Ser^{37} → Phe^{37} (S37F) substitution, was identified in the 888 mel cell line as a melanoma-specific antigen recognized by tumor-infiltrating lymphocytes (*8*). Because it was possible that this mutation increased the stability of β-catenin, we determined β-catenin concentrations in these cells and in 25 other melanoma cell lines. Seven of the lines, including the 888 mel cell, contained elevated amounts of β-catenin relative to normal human neonatal melanocytes (NHEM) (Fig. 1A). Two of the seven appeared to have APC alterations as well: the 1335 mel cells contained a truncated APC and the 928 mel cells had no detectable APC. The truncated APC was not immunoprecipitated by antibody specific to the COOH-terminal sequence of APC, suggesting it was a COOH-terminal truncation similar to that observed in colon cancers (Fig. 1B).

A substantial amount of β-catenin was coimmunoprecipitated with wild-type (WT) APC from five other lines with high levels of β-catenin. The accumulation of β-catenin on WT APC is characteristic of β-catenin stabilization, as has been observed in particular with NH$_2$-terminal deletion mutants of β-catenin (*5*). The 1088 mel cell appeared to contain a truncated β-catenin that accumulated on the APC protein. Another characteristic of stabilized β-catenin is its migration in a monomeric pool upon size fractionation chromatography (*5, 9, 10*). All of the melanoma cells with elevated amounts of β-catenin exhibited a substantial pool of monomeric β-catenin (Fig. 1C). In addition, two of the cell lines with normal amounts of β-catenin, the 1280 and 1300 mel, also contained some monomeric β-catenin.

Up-regulation of β-catenin in the 928 and 1335 mel cell lines may have resulted from loss of WT APC, as has been proposed for colon cancer cells (*4*). To test this hypothesis, we transiently expressed WT APC in the 928 mel cells and costained them with antibodies specific to APC and β-catenin. The 928 mel cells that were positive for ectopically expressed APC contained low concentrations of β-catenin relative to nontransfected cells, which exhibited excessive nuclear and cytoplasmic staining (Fig. 2). The ability of APC to down-regulate β-catenin in the 928 mel cells suggests that they contain WT β-catenin. In contrast, ectopic expression of WT APC in the 888 mel cells did not down-regulate the endogenous mutant β-catenin, but instead resulted in its

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