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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 $Ser^{37} \rightarrow Phe^{37}$ (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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accumulation on the WT APC.

The *wnt-1* proto-oncogene activates β-catenin signaling by reducing the rate of β-catenin degradation (*3*), whereas the APC tumor suppressor enhances this rate (*4*). To examine whether the high steady-state amount of β-catenin in the melanoma cells was due to a reduced rate of turnover, we performed pulse-chase analysis of β-catenin on representative cell lines. The β-catenin in the SK23 mel cell line, which contains WT APC and normal amounts of β-catenin, had a half-life ($T_{1/2}$) of less than 30 min (Fig. 3A). In contrast, the β-catenin in the 888 mel cells, which contained the S37F mutation, had a $T_{1/2}$ of >4.5 hours. The β-catenin in the 928 mel cells, which lack WT APC, and in the 624 mel cells, which contain a mutant β-catenin (Table 1), also had an extended $T_{1/2}$. The 888 mel cells contain mRNAs for both WT and mutant β-catenins (*8*), but the relative contribution of their products to the half-life analysis is unknown. The results suggest that the WT β-catenin is a minor fraction of the total or that the mutant form dominantly interferes with the turnover of the WT protein. The 1088 mel cells contain both a full-length β-catenin with an intermediate $T_{1/2}$ of ~2 hours and a truncated β-catenin with an extended $T_{1/2}$ of >4.5 hours.

To ensure that substitution of Ser^{37} was responsible for the reduced rate of protein turnover, we transfected murine pituitary ATT20 cells, which exhibit rapid turnover of endogenous β-catenin (*5*), with plasmids encoding epitope-tagged $Ser^{37} \rightarrow Ala^{37}$ (S37A) or WT β-catenin. The exogenous WT β-catenin had a $T_{1/2}$ of ~40 min, whereas the S37A β-catenin had a $T_{1/2}$ of >4 hours (Fig. 3B). To determine if the S37A β-catenin was responsive to APC-dependent turnover, we coexpressed it with an *APC25* cDNA in SW480 human colon cancer cells that contain only truncated APC. The APC25 fragment down-regulates β-catenin, whereas the control APC3 fragment does not (*4*). Recovery of the epitope-tagged β-catenins revealed that WT, but not the S37A β-catenin, was degraded in response to the coexpressed APC25 fragment (Fig. 3C). These results demonstrate that a single point mutation has a marked effect on the $T_{1/2}$ of β-catenin.

Sequencing of β-catenin cDNAs from the other melanoma lines with β-catenin accumulation revealed three additional point mutations affecting serine residues (Table 1). As with the 888 mel cells, the mutations identified in the 501 and 1241 mel cells were C to T transitions that produced an S37F substitution. Interestingly, C-to-T transitions are also common in the *p53* gene in melanomas, and may be an effect of ultraviolet radiation (*11*). The mutation in 624 mel predicts $Ser^{45} \rightarrow Tyr^{45}$ (S45Y) substitution, and pulse-chase analysis of this cell suggests that it may prolong the $T_{1/2}$ of β-catenin (Fig. 3). Moreover, coexpression of an S45Y β-catenin with

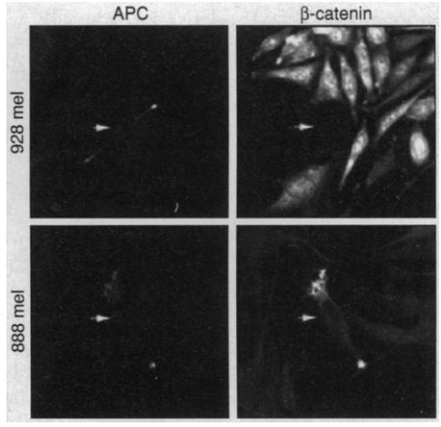

Fig. 2. Down-regulation of β-catenin by ectopic expression of WT APC. The 928 mel and 888 mel cells were transiently transfected with a plasmid encoding human WT APC, and 48 hours later, cells were fixed and costained with anti-APC (left) and anti-β-catenin (right) (*18*).

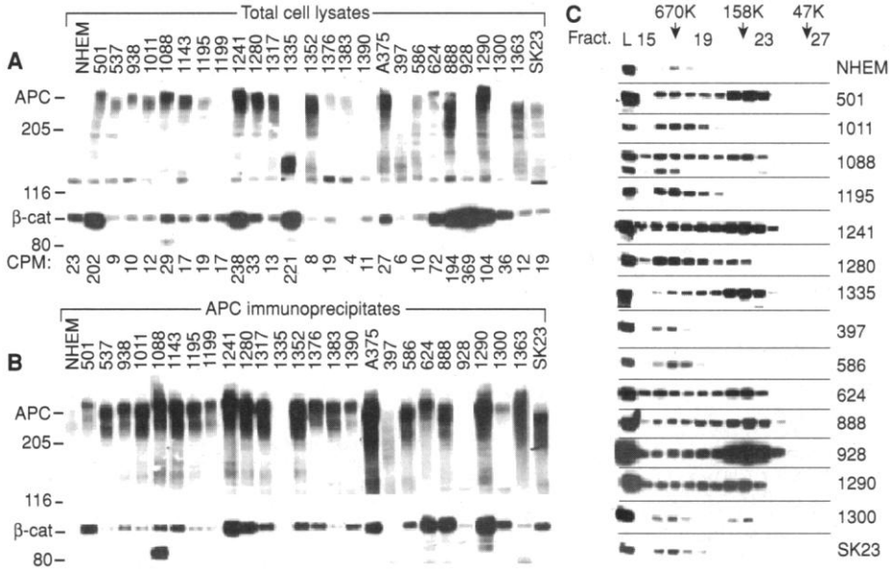

Fig. 1. Analysis of β-catenin and APC in melanoma cell lines. (**A**) Protein-equivalent amounts of total cell lysate from the indicated cell lines were subjected to SDS-PAGE and immunoblotting (*13*). The blot was cut horizontally and developed with anti-APC2 (APC; top) or anti–β-catenin (β-cat; bottom). The β-catenin blot was developed with ^{125}I-labeled protein A, and the counts per minute (CPM) for each β-catenin band is indicated below each lane. For (A) and (B), values at left indicate positions and molecular masses in kilodaltons of protein standards, and NHEM indicates a normal neonatal human melanocyte. All other cell lines were derived from human melanomas (*16*). (**B**) APC was immunoprecipitated from protein-equivalent amounts of the cell lysates, and the precipitates were analyzed for APC and β-catenin with SDS-PAGE and immunoblotting (*13*). (**C**) Size-exclusion chromatography was performed on approximately 800 μg of total protein from each lysate, and fractions were analyzed for β-catenin with SDS-PAGE and immunoblotting. Total lysate (L) and column fraction (Fract.) numbers are shown at top, and arrows indicate the elution positions of protein standards. Longer exposures are presented for cell lines with lower concentrations of total β-catenin.

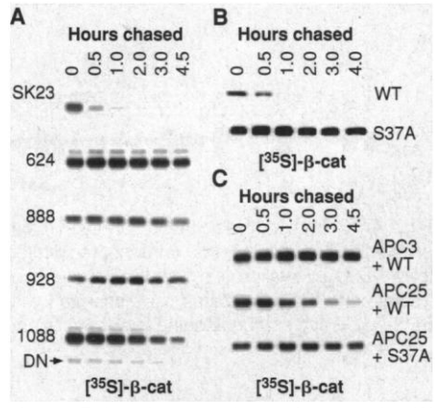

Fig. 3. Pulse-chase analysis of β-catenin. (**A**) Melanoma cells were pulse-labeled with [^{35}S]methionine, chased with cold methionine for the indicated times, and then lysed (*20*). β-Catenin was immunoprecipitated and then analyzed with SDS-PAGE and fluorography. The cell lines are indicated to the left of each panel at the position of the β-catenin band. DN indicates the position of the NH_2-terminal truncated form of β-catenin in the 1088 mel cells. (**B**) ATT20 cell lines stably expressing either WT β-catenin or the S37A mutant were subjected to pulse-chase analysis (*20*). (**C**) SW480 cells were transiently cotransfected with plasmids encoding a COOH-terminal (APC3) or central (APC25) fragment of APC and either the WT or S37A mutant of β-catenin (*20*). APC25 down-regulates β-catenin but APC3 does not (*4*).

APC25 indicated it was refractory to APC-dependent turnover in SW480 cells (*12*). The serines at position 37 and 45 are likely important phosphorylation sites, as the quadruple substitution of Ser^{33}, Ser^{37}, Thr^{41}, and Ser^{45} markedly reduced the phosphorylation of β-catenin in *Xenopus* (*7*). Two novel β-catenin mRNAs, one lacking exons 2 and 3, and the other lacking exons 2, 3, and 4, were identified in the 1088 mel cells. Initiation normally occurs at codon 1 in exon 2; however, initiation at codon 88, the first ATG in exon 4, would account for a truncated β-catenin approximately the size of that detected in the 1088 mel cells (Fig. 1A). A more severely truncated β-catenin, predicted from initiation at codon 174 in exon 5 of the other alternative mRNA, has not been detected. Whether the β-catenin mRNA isoforms in this cell are due to a mutation or to unusual mRNA processing is unclear. None of the other melanoma cells contained these mRNAs. Sequencing of β-catenin cDNAs from the APC-deficient 1335 and 928 mel cells identified only wild-type sequence, as did sequencing of the 1280 mel, 1300 mel, SK23 mel, and NHEM lines.

Recently, β-catenin has been shown to functionally interact with Lef-Tcf transcription factors when overexpressed in *Xenopus* oocytes (*2*). To determine if this interaction occurs in the melanoma cell lines, we immunoprecipitated β-catenin from some of the lines and examined the precipitates for Lef-1. Lef-1 was preferentially coimmunoprecipitated by anti–β-catenin from the cells containing stabilized β-catenin (Fig. 4). This raises the possibility that in these cells a constitutive β-catenin–Lef-Tcf complex may result in persistent transactivation of as yet unidentified target genes.

Of the 26 melanoma cell lines we examined, 8 are defective in β-catenin regulation because of β-catenin mutations, unusual β-catenin mRNA splicing, or inactivation of APC. We hypothesize that these mutations are selected in tumor progression. The mutation in the 888 mel line was unlikely to be generated by in vitro culture, as it was also present in the 1290 mel line, which was derived from a new tumor from the same patient after a 3-year remission (*8*). Moreover, the mutation was also identified in the uncultured tumor material from which the 1290 mel was derived. The stabilizing mutations in β-catenin are also consistent with a proposed function for APC in colon cancer. The ability of WT, but not mutant APC to down-regulate β-catenin in colon cancer cells led us to propose that up-regulation of β-catenin may contribute to cancer progression (*4*). In the melanoma cells, β-catenin mutations were identified in cells that appeared to express WT APC, whereas high amounts of WT β-catenin were found in cells expressing mutant APC. Thus, up-regulation of β-catenin may be a common feature of tumorigenesis that can be effected through mutations in the APC or β-catenin genes or other genes that function in this pathway.

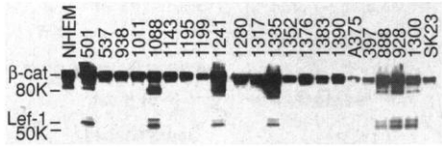

Fig. 4. Coimmunoprecipitation of Lef-1 with β-catenin. β-Catenin was immunoprecipitated from ~600 μg total protein from the indicated cell lysates, and the precipitates were analyzed with SDS-PAGE and immunoblotting for β-catenin and Lef-1 (*13*).

Table 1. Mutations in melanoma cell lines with accumulated β-catenin. AA, amino acid.

Cell line	Nucleotide change	β-catenin AA change
501 mel	TCT → TTT	S37F
1088 mel	Exons 2, 3*	AA 1–87†
	Exons 2, 3, 4*	AA 1–173†
1241 mel	TCT → TTT	S37F
1335 mel‡	Wild type	Wild type
624 mel	TCT → TAT	S45Y
888 mel	TCT → TTT	S37F
928 mel‡	Wild type	Wild type
1290 mel	TCT → TTT	S37F

*Exons deleted from mRNA. †Amino acids deleted. Minimum deletion of amino acid sequence is based on the location of the closest downstream initiator codon. ‡These cells also lack WT APC protein.

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10. This monomeric pool represents unbound β-catenin but does not reflect a lack of association of β-catenin with its binding proteins. For example, cells with this pool of excess β-catenin generally have much higher amounts of β-catenin associated with APC than do those without. There is a 100- to 1000-fold molar excess of β-catenin over APC in most cells and, therefore, saturation of APC with β-catenin would not significantly deplete the monomeric β-catenin pool.
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13. Cell pellets were lysed in Triton X-100 lysis buffer [20 mM tris-HCl (pH 8.0), 1.0% Triton X-100, 140 mM NaCl, 10% glycerol, 1 mM EGTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 mM NaF, 1 mM Pefabloc, and 10 μg/ml each of aprotinin, pepstatin, and leupeptin] and after centrifugation the supernatants were adjusted to a total protein concentration of 2 mg/ml. We applied 25 μl of each sample to 6% SDS-polyacrylamide gel for analysis of total β-catenin and APC by immunoblotting. For immunoprecipitations, 400 μl of each lysate was incubated with 2 μg of affinity-purified polyclonal β-catenin antibody or 2 μg of affinity-purified polyclonal APC3 antibody (*14*). Antibodies were recovered with protein A–Sepharose, and the beads were washed three times with 1 ml each of buffer B [20 mM tris-HCl (pH 8.0), 150 mM NaCl, and 0.5% NP-40] and finally eluted with SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. For immunoblotting, affinity-purified rabbit polyclonal antibody raised against the central region of APC (APC2), full-length β-catenin or full-length Lef-1 (*15*) were incubated with the blots at 0.2 μg/ml. Blots were developed with either the ECL system (Amersham) or, for the β-catenin blot in Fig. 1A, ^{125}I-labeled protein A at 0.5 μCi/ml (Amersham).
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16. The melanoma cell lines were generated from metastatic lesions (*17*) with the exception of the SK23 mel (*21*). The 888 and 1290 mel lines were derived from two independent metastases from the same patient; all others originated from separate patients. The SW480 cell line was obtained from the American Type Culture Collection (ATCC reference CCL228) and is a human colon cancer cell line. ATT20 (ATCC reference CCL89) is a murine pituitary tumor cell. Stable ATT20 clones expressing β-catenins were generated as previously described (*5*).
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18. Transient transfection of 928 mel cells with plasmid encoding human WT APC was performed with lipofectamine (BRL) (*4*). Cells were fixed 48 hours later and stained for immunofluorescence microscopy (*19*). For detection of APC, cells were first incubated with COOH-terminal–specific APC3 antibody (*14*) and then with fluorescein isothiocyanate–conjugated goat antibody to rabbit immunoglobin G (IgG) (Sigma). We detected β-catenin by using mouse anti-β-catenin monoclonal antibody (Transduction Laboratories, Lexington, KY) and Texas red–conjugated donkey antibody to mouse IgG (Cappel, Durham, NC).
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20. Cells were pulse-labeled (*4*) for 30 min and then incubated with media containing unlabeled methionine for the indicated times before lysis on the culture dish. After centrifugation of the lysates, β-catenin was immunoprecipitated from the melanoma cell supernatants with anti–β-catenin, and from the ATT20 or SW480 supernatants by antibody to myc that had been covalently coupled to protein G–Sepharose. Immunoprecipitates were subjected to electrophoresis and fluorography on 8% SDS-polyacrylamide gels. In the transfection experiments (*4*), >50% of the SW480 cells expressed the ectopic cDNA. The APC25 construct encoded APC amino acids 1034 to 2130, and APC3 encoded amino acids 2130 to 2843. For isolation of β-catenin cDNAs, a cDNA pool was first obtained by reverse transcription of total mRNA (RNeasy kit, Qiagen), by use of a mixture of oligo(dT) and random primers. Polymerase chain reaction (PCR) was then performed on the cDNA pool, by use of six distinct primer sets specific for β-catenin cDNA, and the PCR products were cloned into pCR2.1 (Invitrogen) and propagated in *Escherichia coli*. Mutations of β-catenin were confirmed by sequencing analysis of PCR products obtained with the multiple primer sets.

21. We thank M. Waterman for antibody to Lef-1, T. Vuong and J. Heath for assistance with nucleotide sequencing, and T. Boon for SK23 mel cells. Supported in part by Small Business Innovation Research grant 1R43CA69931 from NIH.

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