β-catenin interacts with and inhibits NF-κB in human colon and breast cancer

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Summary

β-catenin plays an important role in development and homeostasis. Deregulated β-catenin is involved in oncogenesis. In this study, we found that β-catenin can physically complex with NF-κB, resulting in a reduction of NF-κB DNA binding, transactivation activity, and target gene expression. Repressed NF-κB activity is found in human colon cancer cells in which β-catenin is activated. Importantly, activated β-catenin was found to inhibit the expression of NF-κB target genes, including Fas and TRAF1. Furthermore, a strong inverse correlation was identified between the expression levels of β-catenin and Fas in colon and breast tumor tissues, suggesting that β-catenin regulates NF-κB and its targets in vivo. Thus, β-catenin may play an important role in oncogenesis through the crossregulation of NF-κB.

Introduction

β-catenin plays two important roles in cells, as a protein associated with E-cadherin in cell-cell adhesion (Ben-Ze'ev and Geiger, 1998), and as a transcriptional activator in Wnt signaling (Willert and Nusse, 1998). Wnt signaling via β-catenin plays a central role in development and homeostasis (Polakis, 2000). A critical control of this pathway is the level of cytosolic β-catenin, which determines the activation of Wnt responsive genes. Without stimulation, β-catenin is degraded by the ubiquitin proteosome pathway, which depends upon β-catenin phosphorylation (Orford et al., 1997). The phosphorylation of β-catenin occurs in a multiprotein complex composed of tumor suppressor protein adenomatous polyposis coli (APC), Axin, and glycogen synthase kinase-3 (GSK-3). In this complex, GSK-3 phosphorylates the N-terminal region of β-catenin, marking β-catenin for ubiquitination-dependent proteolysis (Kikuchi, 1999). Wnt signaling inhibits β-catenin phosphorylation, thus inducing the accumulation of cytosolic β-catenin, which then associates with the TCF/LEF family of transcription factors to activate Wnt/β-catenin-responsive genes (Behrens et al., 1998). Thus, β-catenin phosphorylation controls β-catenin protein level and Wnt signaling.

Defects in Wnt signaling play a major role in human cancers (Korinek et al., 1997; Morin et al., 1997). Inactive mutations of the APC tumor suppressor gene, which is the predominant mechanism leading to β-catenin deregulation, have been reported in approximately 70%–80% of colorectal cancers and a fraction of other types of cancers (Kinzler and Vogelstein, 1996). Mutations in the β-catenin (*CTNNB1*) gene sequences encoding the crucial GSK-3β phosphorylation sites in β-catenin's N-terminal domain have been found in about 10% colorectal cancer, as well as many other different cancer types (Polakis, 2000). The fact that APC mutations and oncogenic β-catenin mutations are mutually exclusive in colon cancer suggests that the major critical consequence of these mutations is the elevation of β-catenin levels in the cytoplasm and nucleus. Deregulation of β-catenin leads to constitutive formation of the β-catenin/TCF complex and altered expression of TCF target genes. Wnt/TCF target genes in cancer cells, which likely cooperate in effecting neoplastic transformation, include c-Myc (He et al., 1998), cyclin D1 (Tetsu and McCormick, 1999), and MMP-7 (matrix metalloproteinase 7/matrilysin) (Crawford et al., 1999).

The transcriptional factor NF-κB is another important signal transduction pathway that participates in the induction of a wide variety of cellular genes involved in immunity, inflammation, and regulation of apoptosis (Bours et al., 2000; Ghosh et al., 1998; Gilmore et al., 1996; Wang et al., 1999). The active complex of NF-κB is composed of two subunits, p65 and p50. Binding sites for NF-κB are present in the promoter region of many genes, such as those encoding cell adhesion molecules, cytokines, and growth factors. NF-κB exists in the cytosol of resting cells bound to inhibitory IκB-α proteins (Baeuerle and Baltimore, 1988). Stimulation with specific inducers, such as TNF or LPS, activates the IκB kinase (IKK) complex that phosphorylates

SIGNIFICANCE

β-catenin is known to play two important functions—as an E-cadherin-associated protein in cell-cell adhesion, and as a transcriptional activator, through its interaction with TCF in Wnt signaling. In this study, we identified a novel role of β-catenin, as an inhibitor of NF-κB. β-catenin exerts this function through physical interaction, which is independent of TCF-mediated transcription. Importantly, β-catenin can repress expression of NF-κB target gene Fas. As deregulated β-catenin and downregulation of Fas play a critical role in tumor progression, the function identified in this study reveals a new direction for understanding the oncogenic roles of β-catenin.

IκB-α, triggering its degradation by the proteosome and allowing free NF-κB to translocate to the nucleus and activate gene expression (Gumbiner, 1995). High levels of NF-κB activity, which are associated with increased resistance to apoptosis induced by TNF and a variety of anticancer reagents, have been shown in diverse solid tumor-derived cell lines (Bours et al., 2000). However, NF-κB plays a dual role in regulation of apoptosis (Bours et al., 2000). NF-κB has been also shown to be essential for Fas expression, to be a proapoptotic molecule (Chan et al., 1999; Zheng et al., 2001), and to be required for p53-mediated apoptosis under some circumstances (Ryan et al., 2000).

Fas (CD95/APO-1) is a cell surface "death receptor" belonging to the tumor necrosis factor receptor (TNFR) family. Fas is constitutively expressed in normal colon epithelial cells which are sensitive to Fas-mediated apoptosis. Fas is downregulated in the majority of colon carcinomas, representing a mechanism for cancer cells to escape lymphocyte-mediated immune surveillance (O'Connell et al., 2000). NF-κB RelA (p65) has been shown to be essential for induction of Fas expression, although strong induction of Fas expression requires combination of multiple cytokines or mitogens (Ouaaz et al., 1999). Thus, in response to TNF-α-induced apoptosis, NF-κB functions as an antiapoptotic molecule. However, NF-κB is required for Fas expression and therefore has a positive role in Fas induced apoptosis. This represents a dual role of NF-κB in regulation of normal tissue homeostasis.

Recently, GSK-3β has been shown to be required for NF-κB activation (Hoeflich et al., 2000). As β-catenin is a major substrate of GSK-3β, it raises an interesting possibility that β-catenin might serve as a mediator for the crossregulation between these two pathways. In this study, we found that β-catenin can physically interact with NF-κB and inhibit its activity. Additionally, suppressed NF-κB activity and NF-κB target gene expression can be found in cancer cells expressing high levels of β-catenin. Importantly, β-catenin was found to inhibit Fas expression through repression of NF-κB. Furthermore, there is a strong inverse correlation of β-catenin and Fas expression in human breast and colon tumor tissues. Thus, this study provides a mechanistic linkage between two important pathways critical for oncogenesis, and also provides a novel mechanism for β-catenin-mediated tumor progression.

Results

β-catenin inhibits NF-κB activity

To investigate the potential effect of β-catenin on NF-κB activity, we first examined the effects of β-catenin on a NF-κB-driven luciferase reporter that can be activated by either p65 or TNF. Cotransfection of human epithelial kidney (HEK) 293 cells with a fixed dose of p65 and increasing doses of either wild-type β-catenin or active mutant β-cateninS37A (Orford et al., 1997) results in a dose-dependent suppression of luciferase activity (Figure 1A), suggesting that β-catenin inhibits p65 transcriptional activity. β-catenin also inhibited the basal level κB-luciferase (in the absence of exogenous p65) to a lesser extent, probably due to suppression of endogenous p65 activity (data not shown). Likewise, TNF-induced NF-κB activity was also suppressed in 293-β-catenin A cells (Figure 1B), 293 cells stably transfected with β-cateninS45Y (another active mutant) (Lin et al., 2000). These observations indicate that β-catenin can inhibit NF-κB transcriptional activity.

To determine whether the suppression of NF-κB transactivation is due to an effect on NF-κB DNA binding, we performed an electrophoretic mobility shift assay (EMSA). We noticed that both the basal level (Figure 1C, lanes 1 and 8) and the TNF-induced level of DNA binding (Figure 1C, lanes 2–3 and 9–10) were significantly reduced in the 293-β-catenin A cells as compared to the parental cells. Quantification of the bands shows about 3- to 5-fold suppression (data not shown). Similar effects were observed with another stable transfectant, 293-β-catenin B cells (data not shown). The inhibition of NF-κB by β-catenin is specific, since the DNA binding of Oct-1, another transcription factor used as an internal control, was not inhibited (Figure 1C, bottom). Thus, β-catenin has an inhibitory effect on NF-κB DNA binding activity.

NF-κB is known to inhibit TNF-induced apoptosis. To determine the effect of β-catenin on this function of NF-κB, we examined its effect in Saos-2 cells, which are sensitive to TNF-induced apoptosis (Ryan et al., 2000). We transfected Saos-2 cells with β-catenin and/or β-catenin plus p65, and then examined the survival cells after TNF treatment. We found that β-catenin enhanced TNF-induced cell death (Figure 1D, lane 3 compared to lane 2). Furthermore, the p65-mediated repression of TNF-induced cell death (Figure 1D, lane 4 compared to lane 2) was derepressed by β-catenin (Figure 1D, lane 5 compared to lane 4), and β-catenin-mediated sensitivity to TNF was reduced by p65 (Figure 1D, lane 5 compared to lane 3), suggesting that the β-catenin-mediated effect is through the NF-κB pathway. Thus, β-catenin is able to exert an inhibitory effect on NF-κB.

β-catenin is known to function as a cotranscription factor for TCF4-directed transcription (Korinek et al., 1997). To determine whether TCF4 signaling is involved, we examined the effect of a dominant negative mutant TCF4 (dnTCF4) (Morin et al., 1997) on β-catenin-mediated inhibition of NF-κB. Using a cotransfection assay, we found that although dnTCF4 abrogated β-catenin-mediated activation of TOP, a reporter known to be activated by the β-catenin/TCF4 pathway (Korinek et al., 1997) (Figure 1E, right panel), it did not affect either β-catenin-mediated inhibition on NF-κB-driven reporter (Figure 1E, left) or p65-stimulated NF-κB reporter (data not shown). Similarily, dnTCF4 was not able to block β-catenin mediated-sensitivity in Saos-2 cells (data not shown). This indicates that β-catenin-mediated inhibition of NF-κB does not go through the β-catenin/TCF4 signaling pathway.

IκB-α is a major regulator of NF-κB. To determine whether IκB-α is involved in this regulation, we examined the effect of β-catenin on IκB-α regulation by immunoblot. We found that overexpression of β-catenin did not effect IκB-α regulation, since there was no difference in either the phosphorylation or the degradation of IκB-α between the 293 cells and the 293-β-catenin cells following TNF treatment (Figure 1F). Therefore, the inhibition of NF-κB activity by β-catenin occurs in an IκB-α- and TCF4-independent manner.

β-catenin complexes with NF-κB

To examine the possibility that β-catenin may inhibit NF-κB through physical interaction, we tested whether β-catenin and NF-κB can form a complex. By a coimmunoprecipitation (IP) assay, we found that both p65 and p50 were able to form a complex with β-catenin, either endogenous β-catenin in the 293

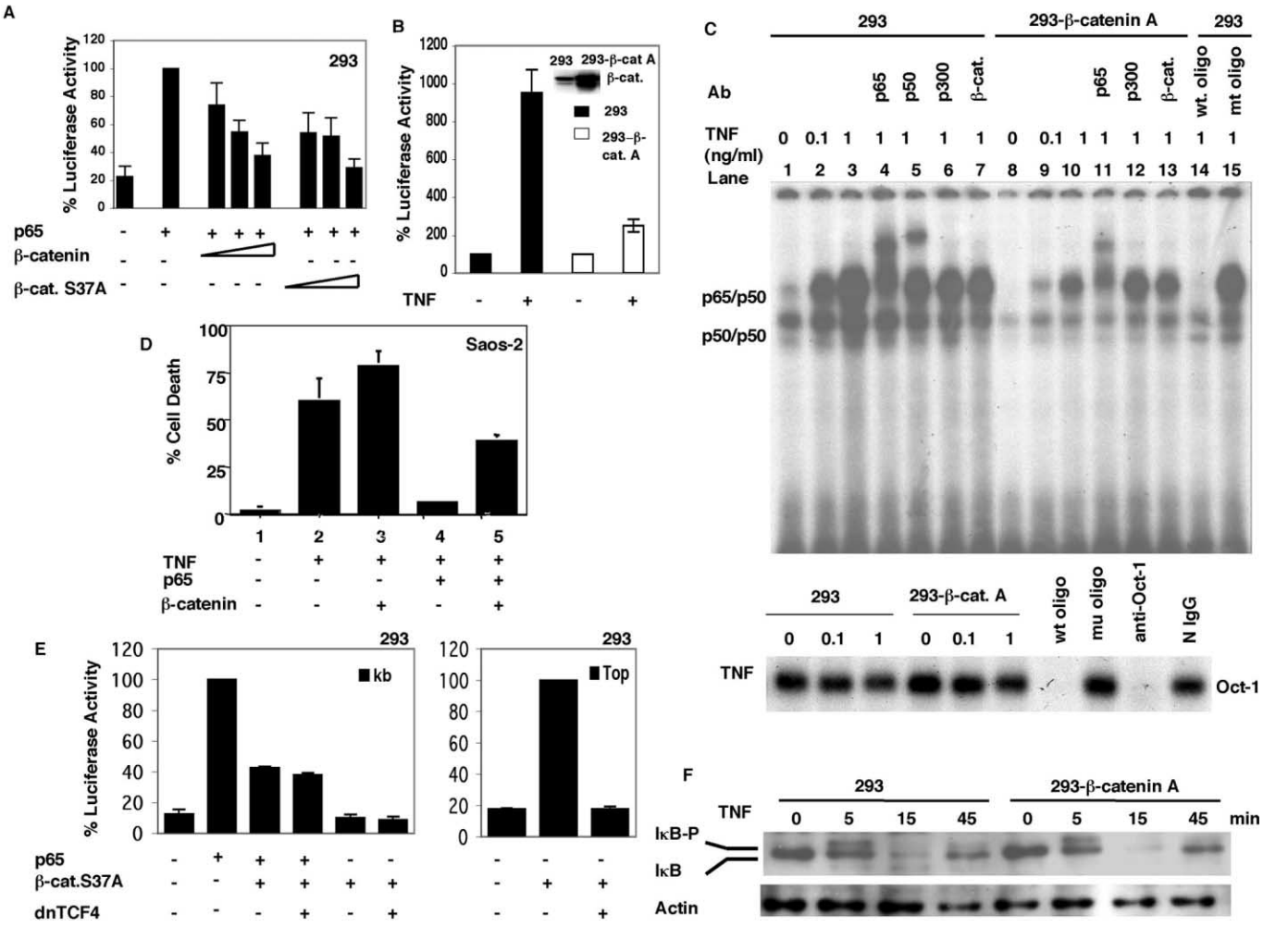

Figure 1. β-catenin inhibits NF-κB transcriptional and DNA binding activities

A: 293 cells were transfected with κB-luc (0.1 μg), p65 (0.2 μg), β-catenin, or active mutant β-cateninS37A (β-catS37A) (0.6, 1.2, 1.8 μg).
B: 293 and 293 β-cateninS45Y stable transfectants (293-β-cat A) were transfected with κB-luc (0.1 μg), with or without treatment of TNF, and basal levels were normalized to 100%. Insert shows β-catenin level by immunoblot.
C: NF-κB DNA binding activity was measured by EMSA. Antibodies against p65, p50, p300, and β-catenin were used for supershifting the complex. Oct-1 DNA binding activity by EMSA used as an internal control.
D: Saos-2 cells were transfected with pcmv-β-gal (0.1 μg), p65 (0.6 μg), IκB-α (0.6 μg), β-catenin (0.6 μg), or p65 plus β-catenin (0.6 μg and 1.2 μg) and then treated with TNF (10 ng/ml) for 24 hr. Percent cell death was calculated from number of viable cells as compared to untreated.
E: 293 cells were transfected with κB-luc (0.1 μg, left) or TOP (0.1 μg, right), p65 (0.2 μg), β-catenin (0.6 μg), and dnTCF4 (1.2 μg). There was no effect by β-catenin or dnTCF4 on the mutant reporter FOP (data not shown).
F: IκB-α level by immunoblot with or without TNF (10 ng/ml) treatment. In all transfection experiments, the total amount of DNA was kept constant with transfection of the empty vector.

vector cells, or stably expressed β-catenin in the 293-β-catenin A and B cells (Figure 2A). This interaction is independent of IκB-α, as it occurred regardless of TNF treatment, which reduced the binding of IκB-α to NF-κB due to IκB-α degradation (Baeuerle and Baltimore, 1988) (Figure 2A). Expression of β-catenin does not change the protein levels of p65, p50 (Figure 2A), or IκB-α (Figure 1F). The interaction was also observed in MCF-7 cells, a breast cancer cell line, suggesting the interaction as a general phenomenon (data not shown). To determine whether β-catenin and NF-κB can directly form a complex, we translated p65 and p50 in vitro, and mixed them with GST-β-catenin protein. By pulling down the GST-β-catenin-interacting proteins using glutathione-agarose, we found that GST-β-catenin does not interact with p65 or p50 alone (Figure 2B). However, in presence of cell lysate, both in vitro-translated, exogenous p65 and p50 were able to bind β-catenin (Figures 2C–2D). This interaction did not occur when the endogenous p65- or p50-depleted cell lysate were used (Figures 2C–2D, insert), suggesting that additional factors that associate with endogenous p65 or p50 are required for the exogenous p65 and p50 to interact with β-catenin. The in vitro-translated p65 and p50 are in natural form, as a p65 antibody can bring down both exogenous p65 and p50 (Figure 2C). The GST-β-catenin is also in natural form, since it can bring down TCF4 protein (Figure 2D). Thus, β-catenin is indeed able to complex with p65 and p50, although additional cellular factors are required.

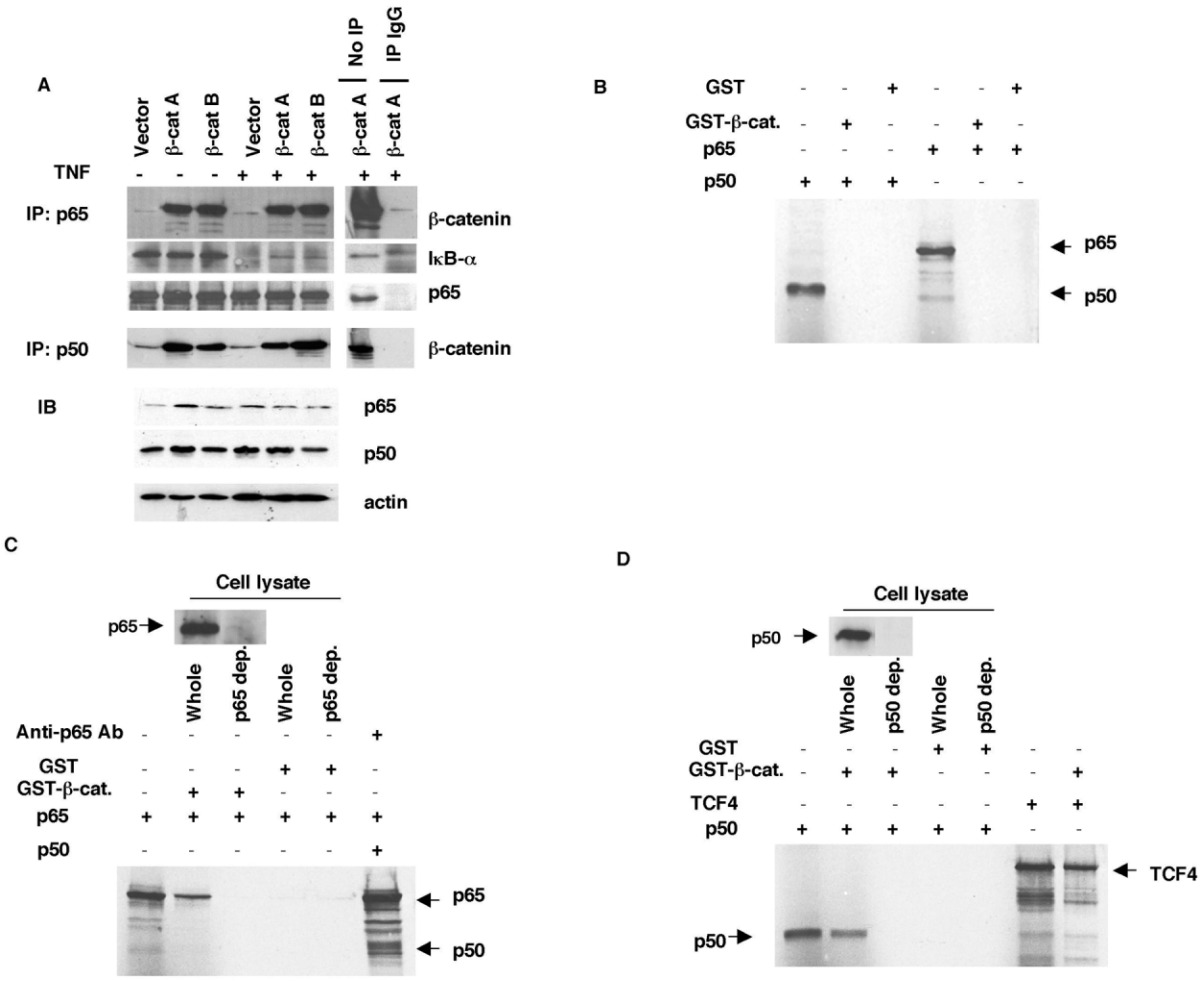

Figure 2. β-catenin physically interacts with NF-κB (p65 and p50)

A: Immunoprecipitation (IP) was performed with antibodies against p65 or p50 (normal IgG as a negative control). The complexes were then subjected to immunoblot as indicated.

B: In vitro translated p65 and p50 (^{35}S-Met labeled) were mixed with GST-β-catenin or GST alone, pulled down by glutathione-agarose, and subjected to SDS-PAGE.

C and D: in vitro translated p65 and p50 proteins were mixed with GST-β-catenin or GST in the presence of whole or p65- or p50-depleted (by repeated IP) cell lysates (RKO), and analyzed as in (**B**); inserts show loaded protein.

Since β-catenin physically associates with NF-κB components, we also asked whether β-catenin is directly involved in the NF-κB-DNA complex. However, a supershift assay by EMSA does not support this notion, as the β-catenin antibody does not affect the migration of the NF-κB-DNA complex (Figure 1C, lanes 7 and 13). Another molecule, p300, known to form complex with both NF-κB (Gerritsen et al., 1997; Perkins et al., 1997) and β-catenin (Hecht et al., 2000; Miyagishi et al., 2000; Sun et al., 2000), also does not seem to be involved, as there is no effect in the DNA binding with p300 antibody (Figure 1D, lanes 6 and 12). Thus, β-catenin may interact with NF-κB and disrupt the ability of NF-κB to bind to the DNA.

NF-κB is suppressed in colon cancer cells

β-catenin is frequently activated in colon cancer cells (Korinek et al., 1997; Morin et al., 1997). As β-catenin inhibits NF-κB, we asked whether NF-κB activity could also be reduced in colon cancer cells in which β-catenin is activated. We examined four colon cancer cell lines, three of which have a high level of β-catenin (HCT116, DLD-1, and SW480), and one of which has a low level of β-catenin (RKO) (Figure 3A). We found that TNF-induced NF-κB DNA binding activity was dramatically lower in cells expressing high levels of β-catenin (Figure 3B, top, lanes 3–8) compared with that in cells expressing low levels of β-catenin (Figure 3B, top, lanes 1–2). The suppression is specific, since there was no significant difference in the control (Oct-1) DNA binding (Figure 3B, bottom). Similarly, NF-κB transcriptional activity in response to TNF, as measured by a NF-κB-driven luciferase assay, was also lower in colon cancer cells expressing high levels of β-catenin (Figure 3C). The reduced NF-κB activity appears to correlate well with the increased β-catenin expression, since the other NF-κB family (p65, p50, and IκB-α) protein levels are similar among these cells (Figure 3A). To determine whether the downstream targets of NF-κB were also affected, we also examined the expression of TRAF1, a downstream target gene of NF-κB (Wang et al., 1998), in

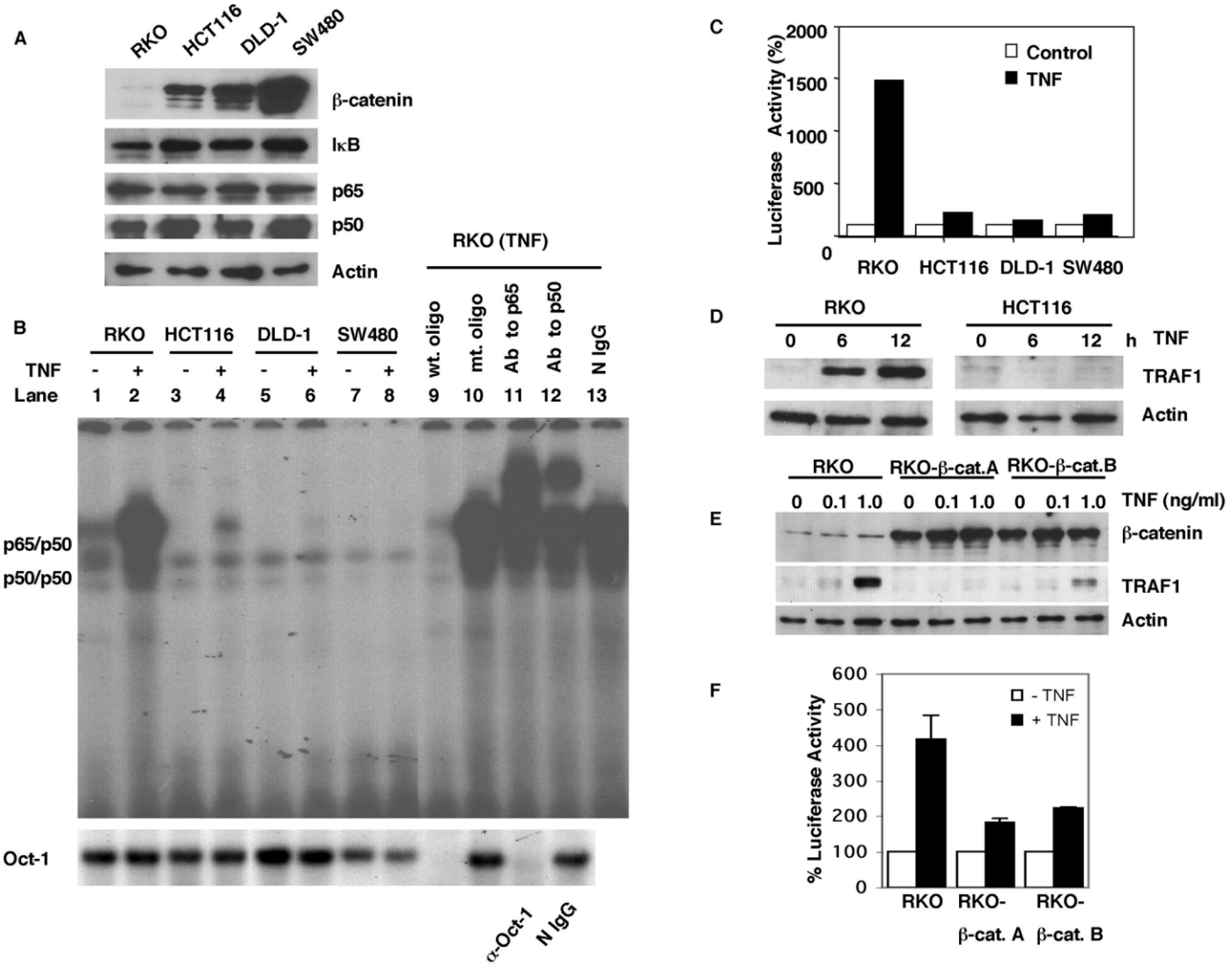

Figure 3. Reduced NF-κB activity in human colon cancer cells that express high level of β-catenin

A: Protein levels of β-catenin, p65, p50, and IκB-α in colon cancer cell lines by immunoblot.
B: Top, EMSA analysis of NF-κB DNA binding activity. TNF treatment was at 10 ng/ml for 15 min. Bottom, EMSA of Oct-1 used as an internal control. Lanes are the same as in NF-κB gel shifting unless specified by antibody used as indicated.
C: Colon cancer cell lines were transfected with κB-luc (1.0 μg) and then treated with TNF (1 ng/ml, 9 hr).
D and E: Immunoblot analysis of NF-κB target gene TRAF1 (TNF concentration for E was 1 ng/ml).
F: RKO and RKO-β-cat cells were transfected with κB-luc (0.3 μg) and treated with TNF as above.

response to TNF. Consistent with DNA binding activity and NF-κB driven reporter, TNF treatment induced a significant amount of TRAF1 expression in RKO cells in a dose- (data not shown) and time-dependent manner (Figure 3D). Contrarily, TNF treatment failed to induce TRAF1 expression in HCT116 cells (Figure 3D), and failed to further enhance TRAF1 expression in DLD-1 and SW480 cells, although there was a basal level of TRAF1 expression in these two cells lines (data not shown). To determine whether the effect was indeed due to β-catenin, we further characterized the role of β-catenin on NF-κB in RKO and HCT116 cells by both overexpression and depletion systems. First, we established RKO-β-cateninS37A stable transfectant cells, and then examined TNF-induced TRAF1 expression. We found that TRAF1 expression was greatly inhibited in these stable transfectant cells (Figure 3E). Moreover, TNF-induced NF-κB driven reporter (Figure 3F) and NF-κB DNA binding activities (data not shown) were also greatly inhibited. Thus, overexpression of β-catenin is able to inhibit NF-κB activity and its downstream target gene expression.

Next, we asked whether β-catenin is required for NF-κB activity. To this end, we characterized the effects of depletion of β-catenin in β-catenin-overexpressing cells, HCT116, which exhibit a high level of β-catenin and suppressed NF-κB activity (Figure 3). HCT116 cells were sensitive to TNF-induced cell death, as TNF treatment dramatically reduced survival cells (Figure 4A). Introduction of GFP-p65, but not GFP as expected (Schmid et al., 2000), inhibited TNF-induced cell death (Figure 4A). Introduction of GFP-p65 also induced κB-driven reporter

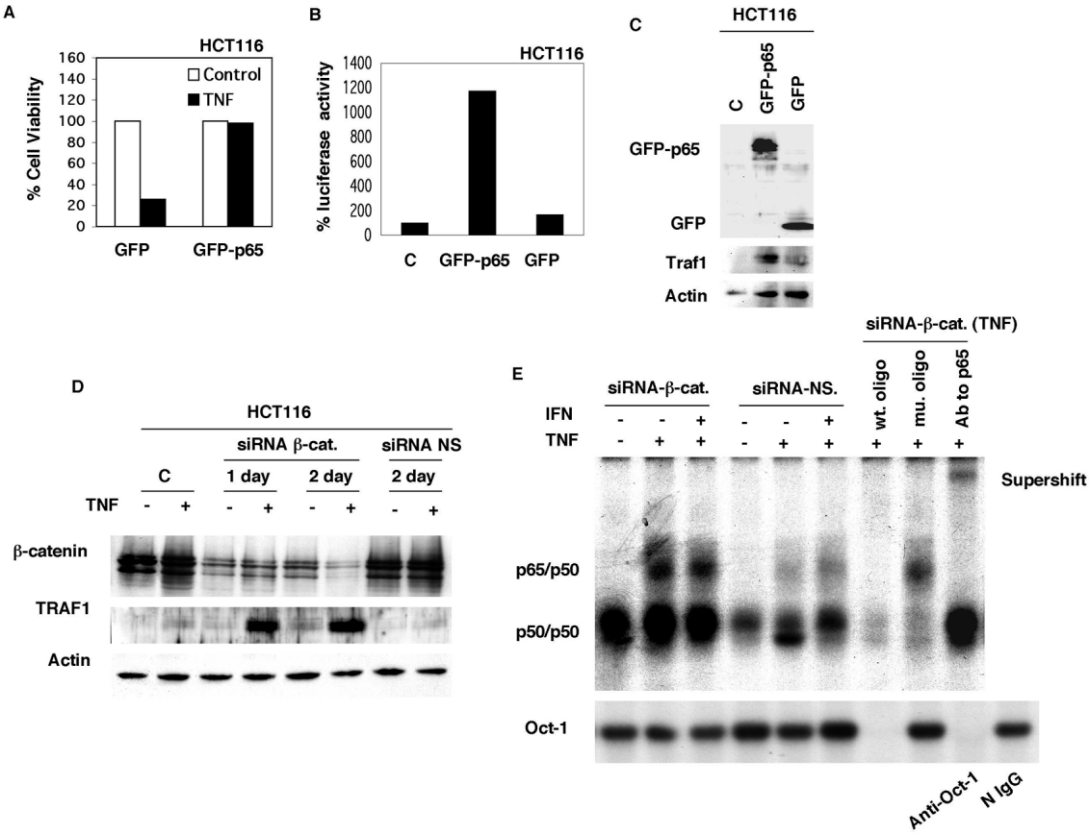

Figure 4. Depletion of β-catenin restores NF-κB activity

A: HCT116 cells were transfected with β-Gal (0.2 μg) and GFP-p65 or GFP (1.6 μg). Two days later, cells were treated with TNF for 24 hr. Percentage of β-Gal-positive cells was counted as surviving cells (viability).

B: HCT116 cells were transfected with κB-luc reporter (0.2 μg) with vector, pGFP-p65, and pGFP (1.6 μg).

C: Immunoblot of cell lysates from HCT116 cells transfected with GFP-p65 or GFP (1.6 μg) or none. Antibodies used in the assay were as indicated.

D: Immunoblot of cell lysates of cells transfected with siRNA-β-cat (1.6 μg) or siRNA-NS (1.6 μg) for 1 or 2 days as indicated (see Experimental procedures). Before harvesting, cells were treated with TNF (10 ng/ml) for 9 hr.

E: EMSA analysis of NF-κB (top) and Oct-1 (bottom) DNA binding activity. Nuclear extracts from cells transfected with siRNA-β-cat or siRNA-NS for 2 days, then TNF treatment (10 ng/ml) or TNF plus INF-γ (10 ng/ml) for 15 min.

activity (Figure 4B) and TRAF1 expression (Figure 4C). Thus, p65 is able to activate its downstream target gene and induce resistance to TNF-mediated apoptosis in HCT116 cells, which overexpress β-catenin. We then used RNA interference to deplete β-catenin in HCT116 cells and examined its effect on NF-κB activities. We transfected small interfering RNA-targeting β-catenin mRNA (siRNA-β-cat) or nonspecific siRNA (siRNA-NS) into HCT116 cells, and then examined the effect on NF-κB activity and its downstream target gene expression. We found that treatment of HCT116 cells with siRNA-β-cat, but not siRNA-NS, resulted in a reduced level of β-catenin, indicating that depletion of β-catenin by siRNA method was effective (Figure 4D). Importantly, TNF stimulation was able to induce the NF-κB target gene TRAF1 expression in cells treated with siRNA-β-cat, but not in those treated with siRNA-NS (Figure 4D). This result indicates that depletion of β-catenin by siRNA restored NF-κB activation in response to TNF stimulation in the β-catenin-overexpressing HCT116 cells, suggesting that suppressed NF-κB is a result of β-catenin overexpression. This conclusion was further supported by EMSA analysis, in which NF-κB DNA binding activity was increased in the cells treated with siRNA-β-cat as compared to cells treated with siRNA-NS after stimulation with TNF or TNF plus IFN-γ (Figure 4E). The effect of siRNA-β-cat on NF-κB was specific, since it did not affect the DNA binding activity of another transcription factor, Oct-1 (Figure 4E, bottom). These results indicate that depletion of β-catenin in colon cancer cells expressing high levels of β-catenin can release the suppression of NF-κB activity and restore the inducible expression of NF-κB target genes. Taken together, the above observations indicate that β-catenin is able to exhibit an inhibitory effect on NF-κB activity and its downstream target gene expression.

β-catenin inhibits Fas expression

To investigate the physiological relevance of this crossregulation, we examined the relationship of β-catenin with another important NF-κB-regulated gene, Fas (CD95). Fas is a member of TNF receptor family and expressed at high level in normal colonic epithelial cells. In colon cancer cells and breast cancer cells, Fas was frequently found downregulated, which may contribute to resistance to Fas-mediated apoptosis (von Reyher et al., 1998), and was proposed to allow cancer cells to escape

from lymphocyte-mediated immune surveillance (O'Connell et al., 2000). However, the mechanism of Fas downregulation is unclear. The p65 (RelA) subunit of NF-κB is known to be required for Fas expression (Ouaaz et al., 1999). Since activation of β-catenin is also frequently found in colon and breast cancers (Morin et al., 1997; Lin et al., 2000), and β-catenin can inhibit NF-κB activity, it raises an interesting possibility that β-catenin may inhibit NF-κB activity and result in the downregulation of Fas in cancer cells, thus allowing cancer cells to escape Fas ligand-induced cell death by lymphocyte. To investigate this possibility, we wanted to confirm that NF-κB is required for Fas expression by comparing Fas express level between MEF wild-type and MEF p65$^{-/-}$ cells. Fas expression level is much lower in p65 knockout MEF cells than in wild-type MEF cells (Figure 5A), indicating that the RelA subunit is indeed required for Fas expression. We then compared the expression levels of β-catenin and Fas in a panel of human breast cancer cell lines by immunoblot. We found that there is an inverse relationship between the expression of β-catenin and that of Fas as well as TRAF1 (Figure 5B). In HBL-100 and MDA-MB-231 cells, in which β-catenin expression is low, the levels of Fas and TRAF1 expression are high (Figure 5B); whereas in BT474, 468, and MCF7 cells, in which β-catenin expression level is relatively high, the levels of Fas and TRAF1 are low (Figure 5B). This result implies that high levels of expression of β-catenin may be involved in downregulation of Fas as well as TRAF1 expression. To determine the regulatory relationship of β-catenin on Fas expression, we then examined the Fas expression in RKO and RKO-β-cat cells. We found that the Fas expression level was much lower in RKO-β-cat than in parental RKO cells (Figure 5C, left). Moreover, with treatment of cells with anti-Fas antibody to stimulate Fas-mediated cell death, we found that RKO-β-cat cells were more resistant to Fas antibody-induced cell death than parental RKO cells (Figure 5C, right), supporting the observation that Fas expression was downregulated in these cells (Figure 5C, left). As the increased level of β-catenin in RKO-β-cat cells can suppress NF-κB activity (Figure 3), we asked if the repressed Fas expression was due to suppression of NF-κB in these cells. To this end, we transfected RKO-β-cat cells with GFP-p65 or GFP, and measured the Fas expression on the GFP-positive cells using PE-labeled anti-Fas antibody by FACS analysis. We found that introduction of GFP-p65 increased Fas expression in the RKO-β-cat cells (Figure 5D), indicating that p65 derepresses β-catenin-mediated Fas repression. This result also suggests that the repressed Fas expression in β-catenin stable transfectants is due to suppression of NF-κB (Figures 3E–3F). Thus, β-catenin-mediated repression of Fas expression was at least partially due to an inhibitory effect on NF-κB pathway. Taken together, these results indicate that overexpression of β-catenin is able to downregulate Fas expression by inactivation of NF-κB.

To further characterize the regulatory role of β-catenin on Fas expression, we then examined the effects of depletion of β-catenin on Fas expression in cancer cells expressing high levels of β-catenin. Transfection of HCT116 cells with siRNA-β-cat, which resulted in decreased β-catenin (Figure 5E, left, top insert), was found to increase Fas expression, suggesting that β-catenin may suppress Fas expression in HCT116 cells (Figure 5E, left bottom). The effect is specific, since it was not observed in the cells transfected with siRNA-NS (Figure 5E, left). To determine the biological consequence of this effect, we then examined Fas-mediated apoptosis in these cells. We treated siRNA-β-cat or siRNA-NS transfected cells with anti-Fas antibody and measured Fas-mediated apoptosis by FACS analysis. Consistent with the result from Fas expression, anti-Fas antibody induced an increased apoptosis in cells transfected with siRNA-β-cat as compared to the cells treated with siRNA-NS (Figure 5E, right). This observation suggests that depletion of β-catenin in colon cancer cells expressing high levels of β-catenin restores the expression of Fas and increases the sensitivity of cells to Fas antibody-mediated apoptosis. As depletion of β-catenin resulted in both increased NF-κB activity (Figures 4D and 4E) and Fas expression in HCT116 cells (Figure 5E, left), we asked if repression of Fas expression by β-catenin overexpression is through NF-κB pathway. We then introduced GFP-p65 into HCT116 cells and measured Fas expression in GFP-positive cells. We found that GFP-p65, but not GFP, increased Fas expression in HCT116 cells (Figure 5F), suggesting that repressed Fas expression is the result of suppressed NF-κB in these cells. Thus, inhibition of NF-κB by β-catenin was at least partially responsible for the suppressed Fas expression. Taken together, these results indicate that depletion of β-catenin in colon cancer cells expressing high levels of β-catenin can restore NF-κB activity (Figures 4D and 4E) and Fas expression (Figure 5E), and increase the sensitivity of Fas-mediated apoptosis (Figure 5E).

Downregulation of Fas in primary cancer tissues

Activation of β-catenin has been reported in a number of cancers, such as colon cancer and breast cancer (Polakis, 1999). To further investigate the potential crossregulation of β-catenin on Fas expression in vivo, we then examined the relationship in primary cancer tissues. By immunohistochemical staining, we found a strong inverse correlation between the active β-catenin expression and the reduction of Fas expression (Figure 6 and Table 1). In the tumor tissues, in which β-catenin expression is low and inactive (membrane), Fas expression is high (Case 1 in Figure 6), whereas in tumor tissues in which β-catenin expression is high and active (cytoplasmic and nuclear), Fas expression is low (Case 2 in Figure 6). It should be mentioned that the two images (Case 1 and Case 2) show adjacent spots of the same tumor tissue with antibodies against either Fas or β-catenin by immunohistochemical staining. This indicates that the activation of β-catenin correlates very well with suppression of Fas in the same tumor tissue area, which strongly suggests that activation of β-catenin is involved in the downregulation of Fas expression in vivo. Taken together, an inverse relationship was found between aberrant activation of β-catenin in colon and breast cancers and downregulation of Fas expression in vivo, which suggests that downregulation of Fas might be involved in β-catenin-mediated tumor progression.

Discussion

In this study, we have shown that β-catenin can physically interact with NF-κB (Figure 2) and inhibit its activity (Figures 1, 3, and 4). Importantly, we have also shown that activation of β-catenin is involved in downregulation of Fas (Figure 5), a NF-κB-regulated gene product, suggesting a novel mechanism for β-catenin-mediated oncogenesis; namely, β-catenin inhibits NF-κB activity, downregulating Fas expression, which may allows cancer cells to escape immune surveillance.

β-catenin inhibits NF-κB through physical interaction, which

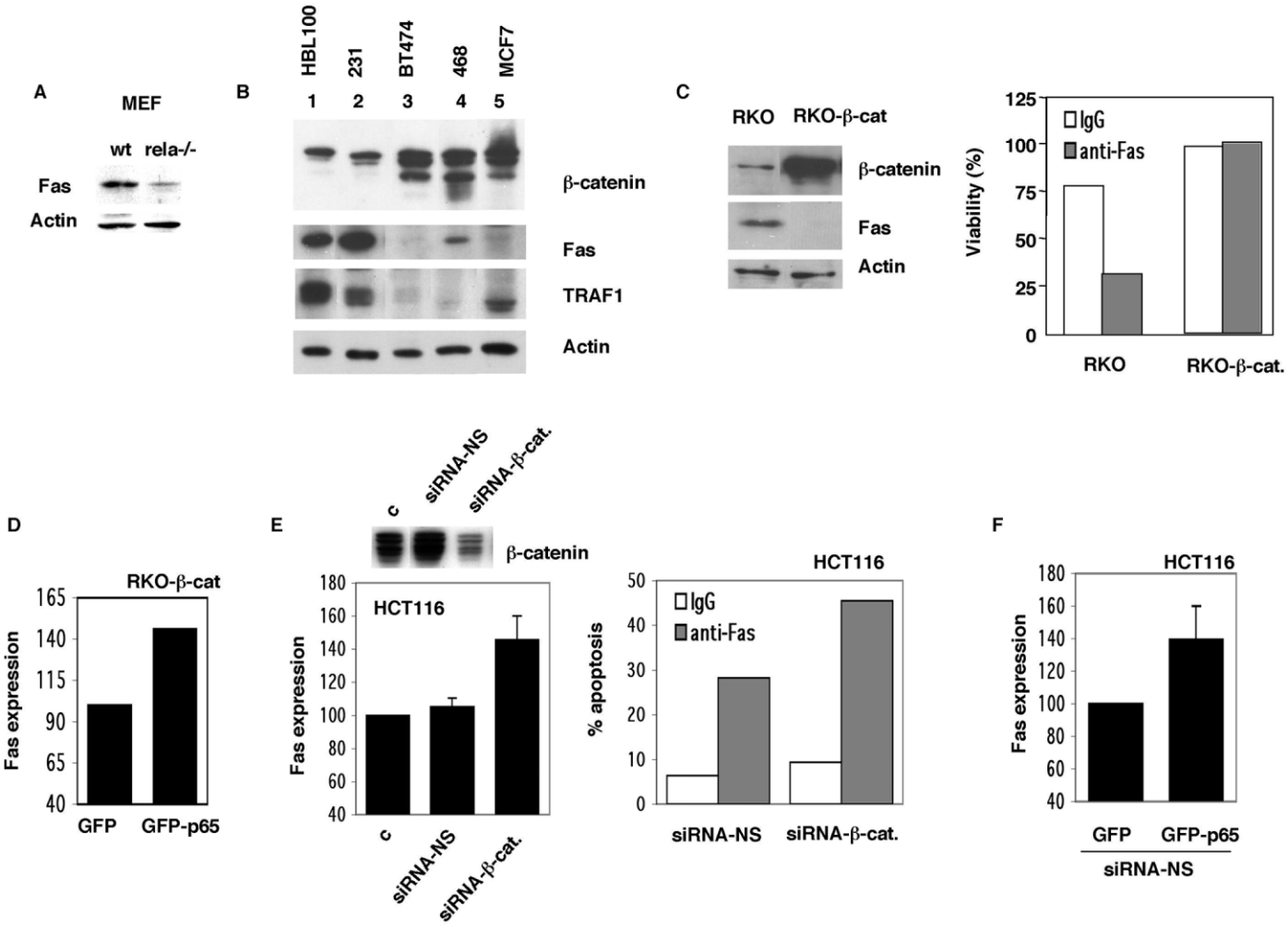

Figure 5. β-catenin inhibits Fas expression

A: Immunoblot of cell lysates from MEF wt or rela$^{-/-}$ cells.

B: Immunoblot of cell lysates from human breast cancer cells with antibodies as indicated.

C: Left, immunoblot of cell lysates from RKO and RKO-β-catA cells. Right, cells were treated with cytokines, IFN-γ (10 ng/ml), and TNF (10 ng/ml) as indicated for 24 hr and then treated with antibody to Fas (CH11) (0.5 μg/ml) or normal mouse IgG (in presence of actinomycin D 5 ng/ml) for another 24 hr. Viable cells were harvested and counted by Trypan Blue exclusion. Bars indicate the percent of surviving cells divided by the untreated control.

D: RKO-β-cat cells were transfected with GFP-p65 or GFP. Two days later, cells were harvested and measured for Fas-expression intensity on GFP-positive cells by FACS analysis.

E: Left, HCT116 cells were transfected with siRNA-β-cat or siRNA-NS for 2 days, then harvested for Fas expression analysis by FACS. Bars show the intensity of Fas expression in each group in comparison with that of control. Right, HCT116 cells were transfected with siRNA-β-cat or siRNA-SN for two days, then treated with anti-Fas antibody (CH11) at 0.5 μg/ml for 24 hr. The percentages of apoptotic cells were derived from analyzing the subG1 population labeled with PI by FACS.

F: HCT116 cells were transfected with GFP-p65 and GFP for two days. Cells were then harvested and assayed for Fas expression as in **D**.

is independent of regulation of the IκB-α (Figures 1F and 2A) and TCF4 signaling pathways (Figure 1E). Thus, in addition to acting as a transcriptional activator for TCF4 and androgen receptor (Yang et al., 2002), β-catenin is also able to act as an inhibitor participating in regulation of other signal transduction pathways. The in vitro protein interaction analysis indicates that the physical interaction between β-catenin and NF-κB is indirect and an additional cellular protein is required (Figures 2B–2D). This implies that the interaction between β-catenin and NF-κB may be subject to another level of regulation. Thus, identification of the interactive domain in β-catenin and the intermediate protein for this interaction, which require further investigation, would be important for understanding the detailed mechanism of the crossregulation by β-catenin on the NF-κB pathway.

Inhibition of NF-κB by β-catenin may provide a plausible mechanism for the observation from the GSK-3β knockout study in which the GSK-3β pathway has been shown to be required for NF-κB activation (Hoeflich et al., 2000). As β-catenin is a major downstream target of GSK-3β, depletion of GSK-3β may lead to an elevated β-catenin expression and suppressed NF-κB activity. However, in the original study, there was no evidence of β-catenin accumulation in GSK-3β (−/−) cells. Although severe cell death was detected in the hepatocyte of the GSK-3β (−/−) mice, it is not clear whether β-catenin might

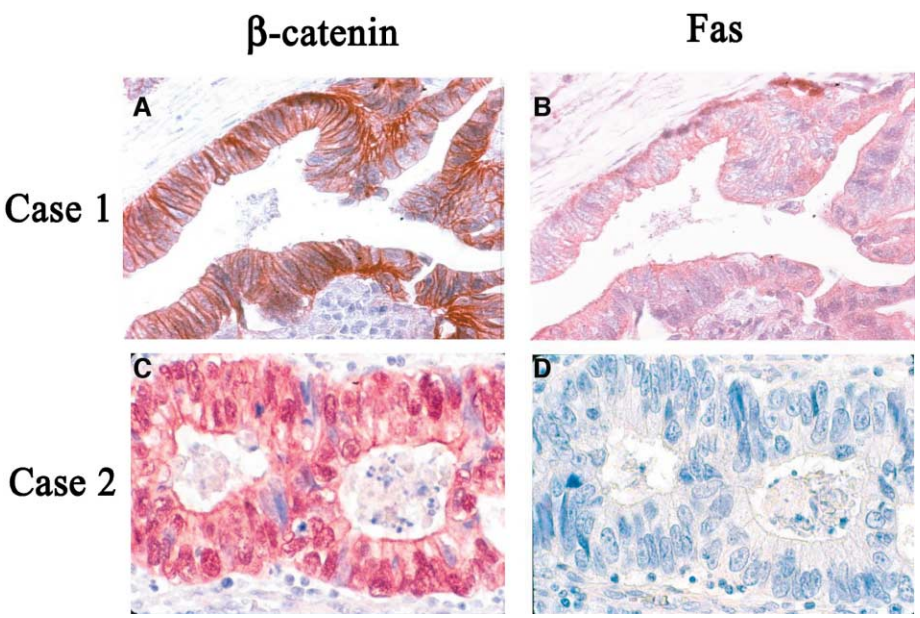

Figure 6. β-catenin inversely correlates with Fas expression in colon cancer

Representative human colon primary tumor tissues samples by immunohistochemistry staining. Case 1, when β-catenin is inactive (primarily in the membrane) (**A**), Fas expression is high (**B**) (two stainings from adjacent areas). Case 2, when β-catenin is active (in the cytoplasm and the nucleus) (**C**), Fas expression level is low (**D**).

contribute to the hepatocyte apoptosis, or whether it could be caused by other mechanisms in GSK-3β (−/−) mice. In our study, we found that β-catenin can inhibit NF-κB. In addition, inhibition of GSK-3β with GSK-3β inhibitors in human colon and breast cancer cell lines increased β-catenin and suppressed both basal and TNF-induced NF-κB activity, suggesting that β-catenin can serve a mediator for inhibition of NF-κB (data not shown). Taken together, our study strongly suggests that β-catenin is a major mediator for the crossregulation of NF-κB by the GSK-3β pathway, although other mechanisms are not excluded.

β-catenin has been shown to be proapoptotic. This property is independent of both TCF4 and the transcriptional function of β-catenin (Kim et al., 2000). However, the mechanism of this observation is not clear. Similarly, the proapoptotic activity of β-catenin has also been observed in vivo. It was shown that overexpression of an activated form of β-catenin in transgenic mice results in a 3- to 4-fold increase of apoptotic cells in the intestinal villi of transgenic mice (Romagnolo et al., 1999; Wong et al., 1998). The results of our study may provide a plausible mechanism for these observations. β-catenin is also implicated to be antiapoptotic in a different system. A recent report demonstrates that Wnt-1, which inhibits GSK-3β and increases β-catenin, can inhibit cellular sensitivity to apoptosis induced by two anticancer agents, vincristine (VCR) and vinblastine (VBL) (Chen et al., 2001). However, this activity requires β-catenin/TCF-mediated transcription, suggesting a different pathway is involved.

NF-κB has been shown to mediate inhibition of TNF- and other anticancer drug-induced apoptosis (Liu et al., 1996; Wang et al., 1996). Aberrant activation of NF-κB has also been found in various types of cancers, which associate with malignant and apoptosis-resistant properties (Bours et al., 2000). In this study, we found that β-catenin inhibits NF-κB. However, as NF-κB exhibits a dual role in regulation of apoptosis and oncogenesis, the phenotypic effects or biological consequences of β-catenin activation may be dependent on the cellular context and/or combined with the activation of particular signaling pathways. For example, it has been recently shown that depletion of NF-κB in the *rela* (NF-κB) knockout mice results in a malignantly transformed phenotype of embryonic fibroblast cells regardless of increased sensitivity to TNF (Gapuzan et al., 2002). It should be mentioned that many protooncogenes, such as ras (Cheng and Meinkoth, 2001; Trent et al., 1996), c-*myc* (Eischen et al., 2001; Hsu et al., 1995), and E2F-1 (Hunt et al., 1997; Macleod, 1999), have all been shown to have a proapoptotic activity when activated and/or overexpressed. As these genes also play a regulatory function in normal cells, association of a proliferative activity with proapoptotic activity may be normal for host to maintain homeostasis. Thus, the association of proapoptic and oncogenic activities by β-catenin may not be a surprise.

Fas is expressed in normal epithelial cells in colon and breast tissue, which are sensitive to Fas-induced apoptosis (von Reyher et al., 1998). This mechanism is important in maintaining the homeostasis of these tissues. However, Fas is downregulated or lost in the majority of colon carcinomas, which usually leads to resistance to Fas-mediated apoptosis. Downregulation of Fas is suggested to be an important mechanism for cancer cells, especially in colon cancer, to escape Fas ligand-mediated immune surveillance (Butler et al., 1998). The mechanisms of Fas

Table 1. Summary of primary tumor tissue sample

A: Summary of colon cancer primary tissue ($p = 0.0351$)

	β-catenin$^-$	β-catenin$^+$	Total
Fas$^-$	2 (10.5%)	15 (78.9%)	17 (89.5%)
Fas$^+$	2 (10.5%)	0 (0%)	2 (10.5%)
Total	4 (21%)	15 (78.9%)	19 (100%)

B: Summary of breast cancer primary tissue ($p = 0.0256$)

	β-catenin$^-$	β-catenin$^+$	Total
Fas$^-$	0 (0%)	5 (33%)	5 (33%)
Fas$^+$	7 (46.7%)	3 (20%)	10 (66%)
Total	7 (46.7%)	8 (53%)	15 (100%)

downregulation in different cancers are unclear. The promoter of the Fas gene contains two NF-κB binding sites, which are required for NF-κB-mediated activation. However, NF-κB activation may be required but not sufficient for Fas expression, as a concert of cytokines was required for strong induction of Fas expression (Chan et al., 1999; Ouaaz et al., 1999). In this study, we demonstrated that β-catenin can inhibit NF-κB, which may limit Fas expression. This observation was demonstrated in vitro and strongly supported by analysis in vivo. By both overexpression and depletion systems, we have shown that β-catenin limits the expression of the NF-κB target Fas. Moreover, the expression level of Fas was found to inversely correlate with that of β-catenin in human breast cancer cell lines, and more importantly in primary tumor tissues. Thus, β-catenin may also be able to contribute to tumorigenicity through downregulation of Fas and/or prevention of Fas-mediated apoptosis, a novel mechanism for β-catenin-involved tumor progression. NF-κB RelA (p65) has been shown to be essential but not sufficient for Fas expression in MEF cells. Other members of NF-κB family, such as p50 and c-Rel, are also involved in the regulation of Fas expression (Zheng et al., 2001). Thus, a primary function of NF-κB may be to promote Fas-induced cell death through the enhancement of Fas expression, rather than inhibit cell death through expression of survival genes (Zheng et al., 2001). It should be noted that RelA is required for Fas expression in MEF cells, although it is dispensable for Fas expression in T lymphocytes (Zheng et al., 2001), suggesting a cell type difference. Thus, the detailed mechanism remains to be investigated of how β-catenin affects Fas expression in human colon and/or breast cancers and the role of this function in β-catenin-mediated oncogenesis.

NF-κB plays a dual role in regulating apoptosis. For example, NF-κB has been shown to be required for p53-induced apoptosis and for induction of resistance to TNF-induced apoptosis (Ryan et al., 2000). It is likely that NF-κB is required for induction of a subset of survival genes against TNFR-mediated apoptosis, as well as a subset of the genes essential for p53-induced apoptosis. In this study, we showed that β-catenin plays a dual role in regulating Fas- and TNFR-mediated apoptosis. Moreover, we have shown that this function may go through the NF-κB pathway. The results of this study suggest that NF-κB is at least partially required for β-catenin-mediated dual regulation of both Fas- and TNFR-mediated apoptosis. In fact, depletion of β-catenin in colon cancer HCT116 cells expressing high levels of β-catenin did increase Fas expression and Fas-mediated apoptosis (Figure 5E), whereas overexpression of β-catenin increased the sensitivity of Saos-2 cells to TNF-induced apoptosis (Figure 1D). Because of the dual role in the regulation of apoptosis, disruption of Fas-mediated apoptosis may therefore be particularly important for β-catenin-mediated oncogenesis in tissues, such as colon, in which the Fas pathway plays a major role in maintaining homeostasis. This possibility was strongly supported by the analysis in vivo, as an inverse relationship was identified between β-catenin and Fas expression in both human colon cancers and breast cancers. Because of the dual regulatory role on the outcome in cells, it is likely that the oncogenic role of β-catenin also requires the cooperation of specific oncogenic alterations during tumor progression. For example, deregulated β-catenin was found to cooperate with ras in cell transformation when ARF and p53 are inactively mutated (Damalas et al., 2001). Thus, the biological outcome of the dual role of β-catenin on cell biology, which goes through NF-κB, may be dependent on cellular context and tissue environment.

In summary, we have shown that β-catenin can inhibit NF-κB in an IκB-independent and TCF-4-independent manner. β-catenin forms a complex with NF-κB components (p65 and p50). β-catenin can inhibit NF-κB transactivation, DNA binding activity, and target gene expression. Importantly, activation of β-catenin inhibits expression of Fas, a NF-κB target gene. Moreover, an inverse correlation between β-catenin and Fas expression was identified in both human colon and breast tumor tissues. Thus, the results of this study provide a novel mechanism for the role of β-catenin on tumor progression.

Experimental procedures

Cell culture and transfection assays

All cell lines were grown in Dulbecco's modified Eagle's medium/F12 (Life Technologies, Inc) supplemented with 10% fetal bovine serum. HEK 293 cells, human colon cancer cell lines SW480, DLD-1, HCT116, and RKO (Morin et al., 1997; Sasaki et al., 2000), breast cancer cell lines MDA-MB-231, MCF-7, BT474, and 468, and the immortalized breast cell line HBL100 were used. Transient transfections were performed with NF-κB-driven-luciferase plasmid (κB-luc.) as reporter, and p65, β-catenin, or active mutant β-cateninS37A (β-catS37A), APC, or mutant APC as effectors. The ratio of effectors and reporter was as indicated. 293 β-cateninS45Y stable transfectants (293-β-cat A and B) were as described (Lin et al., 2000). The transfection agents LPD1 or SN liposomes were incubated with DNA in serum-free media for 30 min before adding to cells and incubating for 3 hr. RKO stable transfectants were established by transfection with β-cateninS37A plasmid. Cell lysates for luciferase activity were collected 48 hr after transfection, and cells were treated with TNF (10 ng/ml) 8 hr before harvesting, unless otherwise indicated. In all the experiments, the total DNA is kept constant by addition of the empty vector. Plasmid pRL-tk (Promega) was used as internal control in all transfection assays. Similar results were obtained when repeated with β-galactosidase as an internal control (data not shown). Cell viability was determined either by number of blue cells stained by β-gal or through trypan blue exclusion as indicated in the legends to Figures 1, 4, and 5. Error bars are the mean ± standard error. Apoptosis induction via Fas pathway was performed by using anti-Fas antibody (CH11, Upstate) at 50 ng/ml for 24 hr.

Immunoprecipitation (IP) and immunoblot

IP was performed with 2 μg of antibodies against p65 or p50 (normal IgG as a negative control) in 1.0 mg whole lysate protein (TNF treatment was 30 min, at 10 ng/ml). Samples were first precleared with a nonspecific IgG antibody. Precleared lysates were then incubated with antibody either to p65 or p50 for 1.5 hr, then incubated overnight with protein G agarose. Samples were washed 4 times with phosphate-buffered solution with 0.1% tween and then subjected to immunoblot as indicated. Cell lysates were separated by SDS/PAGE in a 10% acrylamide gel and transferred onto nitrocellulose membrane for immunoblot. Antibodies to p65 (A), p50 (C-19), IκB-α (C-21), TRAF1 (G-20, H132), GFP (FL), and Fas (c-20) were from Santa Cruz. Antibody to β-catenin was from BD Transduction Laboratories.

In vitro translation and GST pull down

^{35}S-Met-labeled p65 was in vitro translated from pBS-p65 (T7) using a TNT T7 quick-coupled translation/transcription system (Promega). ^{35}S-Met labeled p50 proteins were in vitro translated from pBS-p105 (T3) using a TNT T3-coupled reticulocyte lysate system (Promega). pBS-p105 plasmid was first digested with Best EII, which cut at site 1572 before use. Translation from this digested plasmid resulted in a protein product close to p50. In vitro-translated proteins were mixed either with GST-β-catenin or GST (10 μg) in presence or absence of whole or p65- or p50-depleted (by 3 times IP with excess antibody to p65 or p50) RKO cell lysate (100 μg) for 2 hr at 4°C, then pulled down by glutathione-agarose, and subjected to SDS-PAGE (10%) and autography.

Electrophoresis mobility shift assay (EMSA)

Nuclear extracts from normal or TNF-treated cells were used in EMSA with an oligonucleotide probe containing the NF-κB binding site as described (Zhou et al., 2000) or with the Oct-1 binding site as described (Hoeflich et al., 2000). Eighty-fold of cold wild-type or mutant NF-κB oligonucleotides were used for competition in the nuclear extracts of control or TNF-treated (15 min) 293 cells; doses are as indicated. Antibodies used for supershifting the complex were as indicated. Antibodies to p65 (A), p50 (C-19), p300 (C-20), and Oct-1 (C-21) for supershift were from Santa Cruz.

Flow cytometry

Cells were harvested, washed once with PBS, and stained with PE-conjugated mouse anti-Fas (CD95) antibody (DX2) or normal IgG1 (Pharmigen) for 30 min at room temperature. Cells were then washed once with PBS, fixed in 2% paraformaldahyde, and analyzed using Becton Dickinson flow cytometer. For cells transfected with GFP or GFP-p65, GFP-positive cells were analyzed for PE intensity. The mean values of PE intensity minus the background (PE-labeled normal mouse IgG) were used as relative Fas protein expression level.

Immunohistochemical staining

Tissue samples were deparaffinized and then subjected to a gradient of alcohol and washed five times. They were trypsinized in 0.05% trypsin in PBS and treated with 0.3% H_2O_2 in methanol. Then they were treated with 10% horse serum for 30 min. Sections were incubated with primary antibodies overnight, either β-catenin mAb (1:50 dilution) or Fas (Transduction Laboratories, 1:100). Sections were incubated with secondary antibodies Bio-Anti mouse and rabbit IgG (1:200 dilution) for 1 hr and then incubated with avidin biotin-peroxidase complex diluted in PBS and visualized with aminoethyl carbazole chromogen stock solution. The Fisher exact test was used for statistical analysis with $p < 0.05$ as statistical significance for Fas expression.

Small interfering RNA transfection

Small interfering RNA (siRNA) duplex oligo (Dharmacon) targeting β-catenin mRNA (5′ AAG UCC UGU AUG AGU GGG AAC 3′) or a nonspecific duplex oligo as a negative control (5′ AAC AGU CGC GUU UGC GAC UGG 3′) (1.6 μg/35 mm plate or 12.8 μg/100 mm plate) were transfected using Lipofectamine (Invitrogen) at a ratio of 1 μg RNA to 3 μl Lipofectamine. Experiments were performed on day 1 after transfection.

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