

# E-cadherin binding prevents $\beta$ -catenin nuclear localization and $\beta$ -catenin/LEF-1-mediated transactivation

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## SUMMARY

$\beta$ -catenin is a multifunctional protein found in three cell compartments: the plasma membrane, the cytoplasm and the nucleus. The cell has developed elaborate ways of regulating the level and localization of  $\beta$ -catenin to assure its specific function in each compartment. One aspect of this regulation is inherent in the structural organization of  $\beta$ -catenin itself; most of its protein-interacting motifs overlap so that interaction with one partner can block binding of another at the same time. Using recombinant proteins, we found that E-cadherin and lymphocyte-enhancer factor-1 (LEF-1) form mutually exclusive complexes with  $\beta$ -catenin; the association of  $\beta$ -catenin with LEF-1 was competed out by the E-cadherin cytoplasmic domain. Similarly, LEF-1 and adenomatous polyposis coli (APC) formed separate, mutually exclusive complexes with

$\beta$ -catenin. In Wnt-1-transfected C57MG cells, free  $\beta$ -catenin accumulated and was able to associate with LEF-1. The absence of E-cadherin in E-cadherin<sup>-/-</sup> embryonic stem (ES) cells also led to an accumulation of free  $\beta$ -catenin and its association with LEF-1, thereby mimicking Wnt signaling.  $\beta$ -catenin/LEF-1-mediated transactivation in these cells was antagonized by transient expression of wild-type E-cadherin, but not of E-cadherin lacking the  $\beta$ -catenin binding site. The potent ability of E-cadherin to recruit  $\beta$ -catenin to the cell membrane and prevent its nuclear localization and transactivation was also demonstrated using SW480 colon carcinoma cells.

Key words: APC,  $\beta$ -catenin, E-cadherin, LEF/TCF, Wnt signaling

## INTRODUCTION

The adherens junction complex consists of a transmembrane cell adhesion molecule E-cadherin, whose cytoplasmic domain recruits several molecules including  $\alpha$ -catenin,  $\beta$ -catenin and p120<sup>cas</sup> (Aberle et al., 1996; Kemler, 1993).  $\beta$ -catenin binds directly to the intracellular domain of E-cadherin, and binds to  $\alpha$ -catenin, which connects the adherens junction complex with the actin cytoskeleton (Aberle et al., 1994; Hulsken et al., 1994a; Jou et al., 1995).

While cadherin-bound  $\beta$ -catenin is required for cell adhesion, membrane-uncomplexed  $\beta$ -catenin has a function in transducing the Wnt signal from the cell surface to the nucleus. In the current model, reception of the Wnt signal at the cell surface by Frizzled family members results in stabilization of  $\beta$ -catenin, which then accumulates in the nuclei, and together with members of the LEF/TCF family of DNA binding proteins, activates transcription of Wnt responsive genes (reviewed in Cavallo et al., 1997). In the absence of the Wnt signal, cytoplasmic  $\beta$ -catenin rapidly degrades and only membrane  $\beta$ -catenin complexed with cadherins is protected from degradation. Upon reception of the Wnt signal,  $\beta$ -catenin

levels rise; this is primarily due to stabilization against proteolysis of uncomplexed  $\beta$ -catenin in the cell and is independent of E-cadherin expression (Papkoff, 1997; Papkoff et al., 1996). Although the mechanism by which the Wnt signal increases the level of  $\beta$ -catenin in the cytoplasm is not completely clear, evidence from studies on *Drosophila* (Pai et al., 1997; Peifer et al., 1994) and *Xenopus* (He et al., 1995; Yost et al., 1996) suggests that ZW-3/GSK-3 is involved in the degradation of  $\beta$ -catenin, and the Wingless/Wnt signal acts via Dishevelled to inhibit this regulatory mechanism for  $\beta$ -catenin turnover. Phosphorylation of  $\beta$ -catenin is the most likely mechanism for the regulation of its degradation (reviewed in Daniel and Reynolds, 1997). In mammalian systems, GSK-3 (Rubinfeld et al., 1996) appears to act in concert with APC (Gumbiner, 1995; Rubinfeld et al., 1993; Su et al., 1993) and Axin (Behrens et al., 1998; Hart et al., 1998; Ikeda et al., 1998; Sakanaka et al., 1998) to regulate degradation of  $\beta$ -catenin by the ubiquitin-proteasome system (Aberle et al., 1997; Jiang and Struhl, 1998; Orford et al., 1997) and restrict the  $\beta$ -catenin level in the cytoplasm.

Stabilization of  $\beta$ -catenin is most likely the key factor in the transduction of the Wnt signal. The importance of the  $\beta$ -catenin

level in the cytoplasm is underscored by a discovery that several colon carcinoma and melanoma cell lines have elevated levels of  $\beta$ -catenin due to defects in the degradation machinery or to mutations in  $\beta$ -catenin itself (Korinek et al., 1997; Morin et al., 1997; Munemitsu et al., 1995; Robbins et al., 1996; Rubinfeld et al., 1997). Indeed, overexpression of a degradation-resistant form of  $\beta$ -catenin transforms NIH 3T3 cells (Whitehead et al., 1995), showing that  $\beta$ -catenin itself has oncogenic potential. The nuclear  $\beta$ -catenin in APC-mutant colon carcinoma cells appears to be constitutively active, as measured by transcription of a LEF/TCF reporter gene (Korinek et al., 1997). It has been proposed that abnormally high levels of  $\beta$ -catenin in the nucleus lead to an induction of inappropriate target genes, which would then ultimately result in transformation.

As the main binding partner of  $\beta$ -catenin, E-cadherin plays a pivotal role in  $\beta$ -catenin stabilization and function. Their functional complex is necessary for adhesion and the maintenance of epithelial cell layers. E-cadherin expression is often downregulated in tumor progression, which is associated with dedifferentiation and invasiveness of carcinoma cells (Behrens et al., 1989; Frixen et al., 1991; Perl et al., 1998; Vleminckx et al., 1991). Constitutive expression of E-cadherin decreases cell invasiveness (Chen and Obrink, 1991; Frixen et al., 1991; Navarro et al., 1991; Vleminckx et al., 1991) and prevents progression from pancreatic islet cell adenoma to carcinoma (Perl et al., 1998) although the exact mechanism for this suppressive role of E-cadherin in tumor development is not known.

We directly investigated the competitive nature of  $\beta$ -catenin-binding partner interactions and their effects on the level, subcellular localization and transactivation potential of  $\beta$ -catenin in various cell lines including the nontransformed embryonic stem (ES) cells, which express normal levels of E-cadherin and  $\beta$ -catenin. We demonstrated that the loss of E-cadherin in ES cells results in nuclear localization of endogenous  $\beta$ -catenin that has the ability to bind LEF-1 and transactivate. Our results suggest a hierarchy of protein-protein interactions, which may be of biological relevance to development or tumorigenesis, e.g. where expression of E-cadherin is downregulated.

## MATERIALS AND METHODS

### Recombinant proteins

Cloning, expression and purification of the fusion proteins MBP-ECT, -ECT.823, -ECT.884,  $\beta$ -His6 and GST-LEF have been described (Aberle et al., 1997, 1994; Huber et al., 1996a). GST-APC, including the N-terminal, constitutive  $\beta$ -catenin binding sites of human APC (amino acids 958-1207), was amplified by PCR with *Pwo*-DNA polymerase (Boehringer Mannheim) using primers 5'-ACCGGATCCATGAGATCTTCAAATGATAG-3' and 5'-ACGGG-ATCCTCAAGATCTTTTACTGCTTTGTCCAGA-3', and cloned into the *Bam*HI site of pGEX4T1 (Pharmacia). Murine LEF-1 cDNA encoding amino acids 1-295 was amplified by PCR as above with primers 5'-GCCATGGTACCTATG CCCCAACTTCCGGAG-3' and 5'-CCGGAATTCTTAAGGTCTTTTGGGCTCCTG-3'. The PCR product was cloned into the *Kpn*I/*Eco*RI site of the pThioHisA vector (Invitrogen) and sequenced. For expression of the thioredoxin-LEF-1 fusion protein (Trx-LEF), *E. coli* DH5 cells were grown to an OD<sub>600</sub> = 0.5 at 30°C and induced with 1 mM IPTG for 3 hours. Cells were

lysed in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 2 mM imidazole. The lysate was loaded onto a 5 ml Ni<sup>2+</sup>-chelating Sepharose HiTrap column (Pharmacia) connected to a BioCadSprint Perfusion Chromatography System (Perseptive). After extensive washing with 25 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 2 mM imidazole, bound protein was eluted with a 45 ml linear gradient to 25 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.1 M imidazole, at a flow rate of 3 ml/minute.

### Competition experiments

GST-LEF or GST-APC (1  $\mu$ g/ml) were preincubated with the respective competitor protein (1, 3, 9, 27 and 81  $\mu$ g/ml) for 15 minutes before adding recombinant  $\beta$ -catenin (1  $\mu$ g/ml) for an additional 60 minutes. Protein complexes bound to the GST fusion proteins were pulled down with glutathione-agarose beads. After washing five times with 0.1% Triton X-100 in PBS, proteins were eluted with Laemmli sample buffer and analyzed by western blotting.

### Antibodies, immunoprecipitations and western blotting

Anti-E-cadherin (Kasper et al., 1995), anti- $\beta$ -catenin (Transduction Laboratories), anti-HA (Boehringer Mannheim), anti-APC (APC-5, Oncogene Research Products), anti-p120<sup>ctn</sup> (Transduction Laboratories) and anti-M16C peptide LEF-1 antibodies were used for immunoprecipitations and western blotting experiments. Cells were lysed with PBS containing 0.1% Triton X-100 and a protease inhibitor mix (Complete<sup>TM</sup>, Boehringer Mannheim) by scraping from the culture dish and clearing with centrifugation in a microfuge for 6 minutes at 4°C. Antibodies for immunoprecipitation were added to clarified extracts adjusted for protein concentration (BCA Protein Assay, Pierce) and incubated for 1 hour at 4°C, after which the immune complexes were collected by binding to protein A-Sepharose beads for an additional hour at 4°C. After four washes with lysis buffer the immunoprecipitates were subjected to western blotting. Equal amounts of total cell protein were boiled in Laemmli buffer for 5 minutes, separated by SDS-PAGE, electro-transferred to nitrocellulose and incubated with antibodies as described (Huber et al., 1996a). The proteins were detected using an enhanced chemiluminescence (ECL) detection system (Amersham) following the manufacturer's recommendation.

### Affinity precipitations

Confluent cells were lysed in PBS containing 0.1% Triton X-100 and a protease inhibitor mix (Complete<sup>TM</sup>, Boehringer Mannheim). Samples of lysates containing equivalent amounts of protein (BCA Protein Assay, Pierce) were depleted of endogenous glutathione-S-transferase by incubation with glutathione-agarose beads (10% v/v) for 1 hour at 4°C. Precleared lysates were incubated with 2  $\mu$ g of GST fusion protein bound to glutathione-agarose beads for 1 hour at 4°C. The beads were collected by brief centrifugation in a microfuge and washed five times with lysis buffer by recentrifugation. Laemmli sample buffer was added to the washed beads and the eluted samples were analyzed by western immunoblotting.

### Cell culture, transfections and immunofluorescence

E-cadherin<sup>-/-</sup> ES cells and E-cadherin<sup>-/-</sup> ES cells re-transfected with E-cadherin under a constitutive promoter have been previously described (Larue et al., 1996). ES cells were maintained in an undifferentiated state by growing in 60% BRL medium and replating every 2 days. All other cells were grown in DMEM medium supplemented with 10% FCS. The cells were transiently transfected by the calcium-phosphate method. C57MG and Wnt-1-transfected C57MG cells were gifts from J. Papkoff. For immunofluorescence labeling, cells were allowed to settle on collagen-treated coverslips the day before transient transfection with wild-type E-cadherin and with  $\Delta$ E $\beta$ C, the fusion construct of E-cadherin and  $\alpha$ -catenin, which is lacking the  $\beta$ -catenin binding site (kindly provided by A. Nagafuchi). After 1-2 days the cells were washed with PBS, fixed in 4% paraformaldehyde for 20 minutes at room temperature or with

methanol for 7 minutes at  $-20^{\circ}\text{C}$ , again washed with PBS and permeabilized with 0.1% Triton X-100 in PBS for 5 minutes. After washing with PBS, the cells were incubated with primary antibodies against E-cadherin (Kasper et al., 1995) and/or  $\beta$ -catenin (Transduction Laboratories) at 1:500 dilution for 30 minutes at  $37^{\circ}\text{C}$ , followed by washing in PBS and treatment with secondary rabbit-FITC (Boehringer Mannheim) and mouse-CY3 (Sigma) antibodies for 30 minutes at  $37^{\circ}\text{C}$ . The cells were then washed in PBS, mounted on slides in a DAPI-containing mounting medium, and viewed using a Zeiss Axioskop fluorescence microscope.

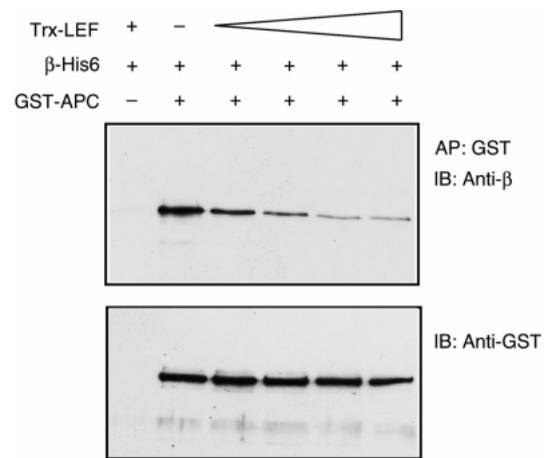
### Transcriptional activation assay

E-cadherin $^{-/-}$  ES cells were grown in BRL-conditioned medium;  $4 \times 10^5$  cells were seeded 1 day prior to transfection on gelatinized 35 mm plates. Cells were transfected by the calcium-phosphate transfection method with 1  $\mu\text{g}$  of pCMV $\beta$ -Galactosidase as an internal standard and 2  $\mu\text{g}$  of TOPFLASH luciferase reporter plasmid (van de Wetering et al., 1997). 2  $\mu\text{g}$  of E-cadherin, E $\Delta\beta\alpha\text{C}$  (Nagafuchi et al., 1994) and S33A  $\beta$ -catenin in a pcDNA3 expression vector were added in various combinations. The expression vector alone was added to compensate for the total amount of DNA per transfection. 1-2 days after transfection, cells were lysed and 20  $\mu\text{l}$  of each sample was taken to determine luciferase and  $\beta$ -Galactosidase activity in an EG&G Berthold Autolumat LB953 by injecting 300  $\mu\text{l}$  luciferase buffer (50  $\mu\text{M}$  luciferin, 2 mM ATP, 10 mM  $\text{MgCl}_2$ , 25 mM Gly-Gly, pH 7.8) or 100  $\mu\text{l}$   $\beta$ -Gal reaction buffer (60 mM  $\text{Na}_2\text{HPO}_4$ , 40 mM  $\text{NaH}_2\text{PO}_4$ , 10 mM KCl, 1 mM  $\text{MgSO}_4$ , 10  $\mu\text{g}/\text{ml}$  Galacton, pH 7.0), followed by 300  $\mu\text{l}$   $\beta$ -Gal Stop Mix (0.2 M NaOH, 5% Emerald Enhancer). The luciferase activity was normalized to  $\beta$ -Galactosidase activity as an internal transfection control. All measurements were done in duplicate and the experiment was repeated five times.

## RESULTS

### LEF-1 and APC form mutually exclusive and competitive complexes with $\beta$ -catenin in vitro

$\beta$ -catenin binds to several protein partners in different subcellular compartments. E-cadherin, LEF-1 and APC can all bind to the central region of  $\beta$ -catenin, with their binding sites partially overlapping (reviewed in Willert and Nusse, 1998). E-cadherin and APC have been shown to compete for interaction with  $\beta$ -catenin (Hulsken et al., 1994b; Rubinfeld et al., 1995). The tumor suppressor gene APC is involved in a rapid breakdown of free  $\beta$ -catenin; the loss of APC function results in increased pools of free  $\beta$ -catenin and in a constitutively active  $\beta$ -catenin/LEF-1 complex (Korinek et al., 1997; Rubinfeld et al., 1997). Therefore, we wanted to determine if LEF-1 and APC proteins actually compete for binding to  $\beta$ -catenin in vitro. To study the complexes of  $\beta$ -catenin with LEF-1 and APC, we chose 293 cells, which contain a large pool of endogenous APC. In addition, we transfected these cells with  $\beta$ -catenin and LEF-1. The cell extracts were immunoprecipitated with antibodies against APC or  $\beta$ -catenin and blotted against  $\beta$ -catenin and LEF-1. Although LEF-1 was found in a complex with  $\beta$ -catenin, it could not be detected in a complex with  $\beta$ -catenin that was co-precipitated with APC (not shown). From this we concluded that LEF-1 binds only to  $\beta$ -catenin that is not complexed with APC. To further demonstrate the mutually exclusive and competitive nature of the LEF-1/ $\beta$ -catenin and APC/ $\beta$ -catenin complexes, we conducted an in vitro competition experiment with recombinant proteins (Fig. 1). APC (GST-APC; amino acids 958-1207) and increasing amounts of LEF-1 (Trx-LEF)

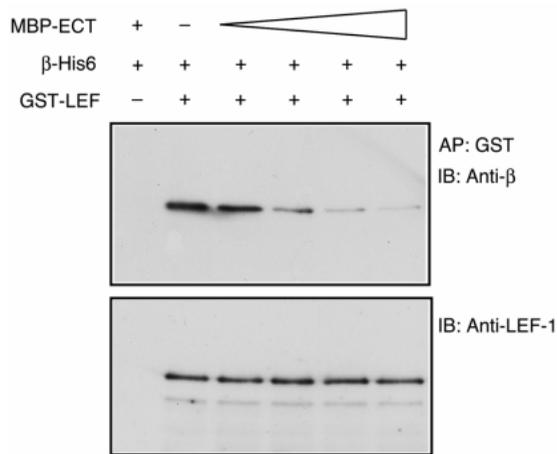


**Fig. 1.** LEF-1 and APC compete for binding to  $\beta$ -catenin. GST-APC and increasing amounts of Trx-LEF recombinant fusion proteins were preincubated together before  $\beta$ -catenin ( $\beta$ -His6) was added. Protein complexes were precipitated with glutathione-agarose beads and the levels of  $\beta$ -catenin determined by blotting with anti- $\beta$ -catenin antibody (upper panel) and with anti-GST antibody as a control for loading (lower panel). Increasing the amount of LEF-1 recombinant protein reduced the amount of  $\beta$ -catenin complexed with APC. AP, affinity precipitation; IB, immunoblotting.

recombinant fusion proteins were preincubated together and then  $\beta$ -catenin ( $\beta$ -His6) was added. Protein complexes were precipitated with glutathione-agarose beads and the levels of  $\beta$ -catenin determined by blotting with anti- $\beta$ -catenin antibody (upper panel) and with anti-GST antibody as a loading control (lower panel). The amount of  $\beta$ -catenin complexed with APC decreased with increasing amounts of LEF-1, indicating that LEF-1 and APC compete for binding to  $\beta$ -catenin.

### E-cadherin and LEF-1 form mutually exclusive complexes with $\beta$ -catenin in vitro and compete for binding to $\beta$ -catenin

Both E-cadherin and LEF-1 bind to  $\beta$ -catenin Arm repeats (Behrens et al., 1996; Huber et al., 1996b; Hulsken et al., 1994b; Orsulic and Peifer, 1996). To examine whether E-cadherin and LEF-1 form shared or separate complexes with  $\beta$ -catenin, we transfected 293 cells with cDNAs for  $\beta$ -catenin, HA-LEF-1 and E-cadherin. The complexes formed were immunoprecipitated with antibodies against E-cadherin and  $\beta$ -catenin and analyzed by immunoblotting with antibodies against E-cadherin,  $\beta$ -catenin and LEF-1. LEF-1 was found in a complex with  $\beta$ -catenin but only with  $\beta$ -catenin that was not in a complex with E-cadherin (not shown), indicating that E-cadherin and LEF-1 form independent complexes with  $\beta$ -catenin. To determine if E-cadherin can compete out LEF-1 in its association with  $\beta$ -catenin, we carried out an in vitro competition experiment using increasing amounts of E-cadherin cytoplasmic tail (Fig. 2). Recombinant LEF-1 (GST-LEF) and increasing amounts of E-cadherin cytoplasmic tail (MPB-ECT) fusion proteins were preincubated, and then recombinant  $\beta$ -catenin ( $\beta$ -His6) was added. GST protein complexes were collected with glutathione-agarose beads and visualized with antibodies against  $\beta$ -catenin and LEF-1. As the amount of the E-cadherin cytoplasmic tail increased, the



**Fig. 2.** E-cadherin and LEF-1 compete for binding to  $\beta$ -catenin. Recombinant E-cadherin cytoplasmic tail (MPB-ECT) and LEF-1 (GST-LEF) fusion proteins were preincubated before adding the recombinant  $\beta$ -catenin ( $\beta$ -His6). GST protein complexes were collected with glutathione-agarose beads and visualized with antibodies against  $\beta$ -catenin and LEF-1. Increased amounts of the E-cadherin cytoplasmic tail resulted in a decreased amount of  $\beta$ -catenin in the complex with LEF-1 (upper panel), showing that E-cadherin cytoplasmic domain can compete out LEF-1 in its association with  $\beta$ -catenin. Anti-LEF-1 antibody was used as a control for loading a constant amount of LEF-1 protein (lower panel). AP, affinity precipitation; IB, immunoblotting.

amount of  $\beta$ -catenin in the complex with LEF-1 decreased (upper panel). Immunoblotting with anti-LEF-1 antibody confirmed that the amount of LEF-1 protein was constant (lower panel). Thus the E-cadherin cytoplasmic domain can prevent binding of LEF-1 to  $\beta$ -catenin.

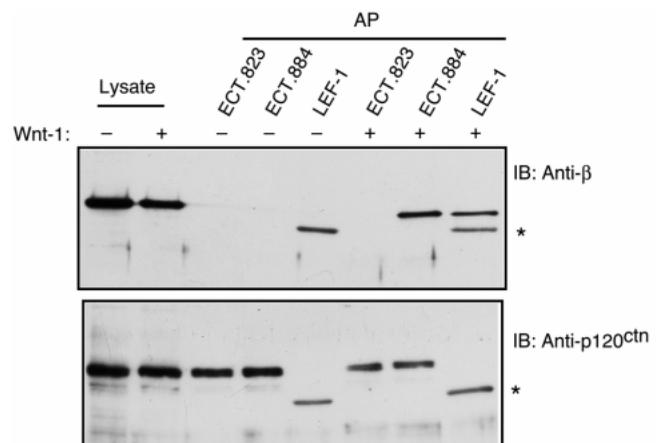
### Only free $\beta$ -catenin that is not associated with the membrane complex binds to LEF-1 in Wnt-1-transfected C57MG cells

Wnt-1 stimulation results in an elevated cytoplasmic level of  $\beta$ -catenin, which can then interact with the LEF-1 transcription factor (Porfiri et al., 1997). To distinguish whether it is exclusively the uncomplexed  $\beta$ -catenin that interacts with LEF-1, we compared the binding of the GST-LEF-1 fusion protein to  $\beta$ -catenin in cell lysates of C57MG cells versus Wnt-1-transfected C57MG cells (Fig. 3), because these cells have already been characterized for their free pool of  $\beta$ -catenin (Papkoff et al., 1996). To selectively recover the uncomplexed pool of  $\beta$ -catenin, we used recombinant GST protein fused to the full-length E-cadherin cytoplasmic tail (GST-ECT), since this fusion protein was previously shown to specifically interact with the uncomplexed  $\beta$ -catenin in the cell but not with  $\beta$ -catenin already complexed with E-cadherin (Papkoff et al., 1996). For *in vitro* affinity precipitation analysis, GST-ECT protein coupled to glutathione-agarose beads was incubated with cell extracts and recovered by precipitation. The precipitates were analyzed by western immunoblotting for the presence of  $\beta$ -catenin and p120<sup>ctn</sup>. ECT.884 contains the full-length E-cadherin cytoplasmic domain (amino acids 737-884). As a negative control we used ECT.823, which contains a truncated E-cadherin cytoplasmic tail (amino acids 737-823) lacking the  $\beta$ -catenin binding site. The full-length E-cadherin

cytoplasmic domain interacted with a significant amount of uncomplexed  $\beta$ -catenin in Wnt-1-transfected C57MG cells, whereas in untransfected C57MG cells no  $\beta$ -catenin was bound (Fig. 3, upper panel). As a control for affinity precipitation, we used p120<sup>ctn</sup>, which binds to a conserved juxtamembrane domain of the E-cadherin cytoplasmic domain (amino acids 737-772). Since the p120<sup>ctn</sup> binding site is more membrane-proximal than the binding site of  $\beta$ -catenin, both the full-length E-cadherin cytoplasmic tail and its truncated form still precipitated p120<sup>ctn</sup> (Fig. 3, lower panel). GST-LEF-1 fusion protein comparably precipitated  $\beta$ -catenin in Wnt-1-transfected cells only, and not in untransfected cells (Fig. 3). Since both Wnt-1-transfected and untransfected C57MG cells contain the complexed  $\beta$ -catenin, we conclude that LEF-1 interacts only with the free pool of  $\beta$ -catenin in Wnt-1-transfected cells.

### Loss of E-cadherin in ES cells results in free $\beta$ -catenin that can interact with LEF-1, thereby mimicking Wnt signaling

Having shown that LEF-1 and E-cadherin form mutually exclusive complexes with  $\beta$ -catenin and compete for the same pool of  $\beta$ -catenin (Fig. 2), we then wanted to determine the fate



**Fig. 3.** Uncomplexed  $\beta$ -catenin interacts with the LEF-1 nuclear factor in Wnt-1-transfected C57MG cells. GST fusion proteins of the murine E-cadherin cytoplasmic domain (ECT) and LEF-1 were used to affinity-precipitate free  $\beta$ -catenin in control C57MG cell lysates (- lanes) and in Wnt-1-transfected C57MG cell lysates (+ lanes). ECT.823 is a fusion protein of GST with a truncated E-cadherin cytoplasmic domain (containing 823 amino acids) that lacks the  $\beta$ -catenin binding site but still has the p120<sup>ctn</sup> binding site; ECT.884 is a fusion protein of GST with a complete E-cadherin cytoplasmic domain (containing 884 amino acids), which includes the  $\beta$ -catenin and p120<sup>ctn</sup> binding sites; GST-LEF-1 encodes full-length LEF-1. No free  $\beta$ -catenin was detected in untransfected C57MG cells. Wnt-1-transfected cells accumulate a free pool of  $\beta$ -catenin, which can be affinity-precipitated with the E-cadherin cytoplasmic domain containing the  $\beta$ -catenin binding site (ECT.884), but not with the E-cadherin cytoplasmic domain without the  $\beta$ -catenin binding site (ECT.823). The precipitates were analyzed for the presence of p120<sup>ctn</sup> as a control for the ability of E-cadherin fusion proteins to associate with uncomplexed proteins (lower panel). LEF-1 specifically precipitates  $\beta$ -catenin only in Wnt-1-transfected C57MG cells, whereas in untransfected cells no available free  $\beta$ -catenin exists for association with LEF-1. Bands marked with an asterisk are nonspecific and appear variably upon incubation with secondary antibody. IB, immunoblotting; AP, affinity precipitation.

of  $\beta$ -catenin in the absence of E-cadherin in nontransformed cells in which E-cadherin is normally the major interacting partner of  $\beta$ -catenin at the membrane. One likely possibility was that the loss of E-cadherin would result in increased availability of free  $\beta$ -catenin, which then could complex with LEF-1. Here we took advantage of genetically altered ES cells, generated by E-cadherin gene targeting, which do not express any detectable E-cadherin (Larue et al., 1996, 1994). Although the steady-state level of  $\beta$ -catenin was higher in wild-type ES cells (which express a large amount of E-cadherin), the E-cadherin $^{-/-}$  ES cells also contained a significant amount of  $\beta$ -catenin (Fig. 4), which must be an E-cadherin-uncomplexed  $\beta$ -catenin (although other cadherins might substitute for E-cadherin at the cell membrane). We used the same biochemical assay as described above for the Wnt-1-expressing cells (Fig. 3) to distinguish between the complexed and free pools of  $\beta$ -catenin in ES cells (Fig. 4). We found that the full-length E-cadherin cytoplasmic tail (ECT.884) only precipitated  $\beta$ -catenin in E-cadherin $^{-/-}$  ES cells and precipitated very little or nothing in the lysates of wild-type ES cells (Fig. 4, upper panel). These results confirm that E-cadherin $^{-/-}$  ES cells do accumulate free  $\beta$ -catenin. In a negative control, a truncated E-cadherin cytoplasmic tail (ECT.823) did not precipitate  $\beta$ -catenin but did precipitate p120<sup>ctn</sup> (Fig. 4, lower panel). LEF-1 precipitated free  $\beta$ -catenin only in E-cadherin $^{-/-}$  ES cell lysates, further confirming that only E-cadherin-uncomplexed

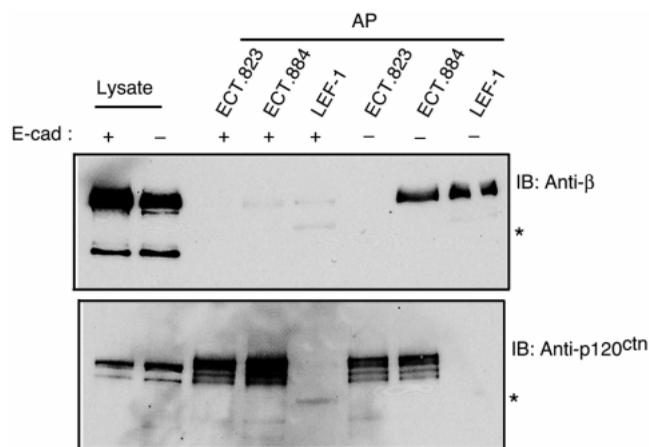
$\beta$ -catenin can interact with LEF-1. In this sense, lack of E-cadherin in mutant ES cells mimics Wnt-1 signaling by upregulating the amount of free  $\beta$ -catenin, which can then form a complex with LEF-1.

### E-cadherin expression prevents nuclear localization of $\beta$ -catenin

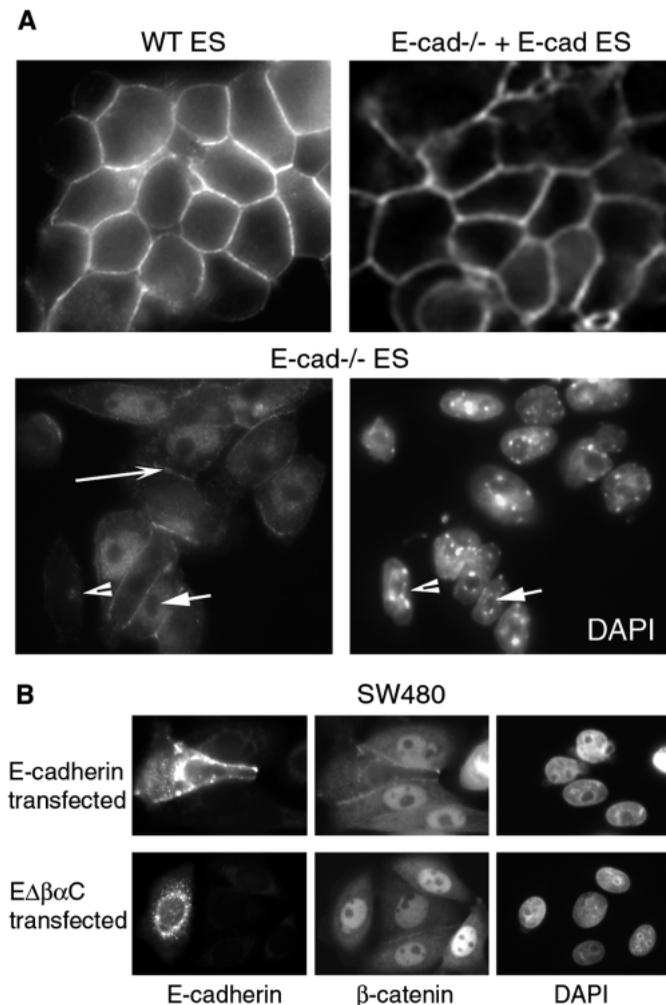
Using immunofluorescence, we examined how the loss of E-cadherin affects the subcellular localization and level of  $\beta$ -catenin in genetically altered E-cadherin $^{-/-}$  ES cells (Fig. 5A). In wild-type ES cells (WT ES), most of the  $\beta$ -catenin is localized at the cell membrane. In E-cadherin $^{-/-}$  ES cells (E-cad $^{-/-}$  ES), some of the  $\beta$ -catenin is still localized at the membrane (Fig. 5A, long arrow), presumably in a complex with N-cadherin, whose expression was found to be slightly upregulated in these cells (data not shown). The presence of other cadherins in these cells may also explain the weak but detectable cell adhesiveness of E-cadherin $^{-/-}$  cells. Aside from the membrane, we observed diffuse  $\beta$ -catenin staining in the nucleus and perinuclear regions, with apparent exclusion from the nucleoli (Fig. 5A, short arrow). In some cells there were aggregates of  $\beta$ -catenin close to the nucleus (Fig. 5A, arrowhead), which colocalized with several Golgi markers (not shown). Immunofluorescence analysis thus confirmed the existence of free, non-membrane-associated  $\beta$ -catenin in the E-cadherin $^{-/-}$  ES cells. E-cadherin $^{-/-}$  ES cells that were retransfected with E-cadherin driven by a constitutive promoter (Larue et al., 1996) localized  $\beta$ -catenin at the cell membrane (Fig. 5A, E-cad $^{-/-}$  + E-cad ES).

Next we wanted to test whether the introduction of E-cadherin is sufficient to 'soak up' the free  $\beta$ -catenin in cells that do not express E-cadherin and that have an even larger pool of free  $\beta$ -catenin than E-cadherin $^{-/-}$  ES cells. We chose SW480 colon carcinoma cells since they have a large pool of uncomplexed  $\beta$ -catenin due to a mutation in the APC gene (Munemitsu et al., 1995; Rubinfeld et al., 1993). Most of the  $\beta$ -catenin in SW480 cells is found in the nucleus. We transiently transfected these cells with E-cadherin cDNA. In approximately 90% of the cells that expressed E-cadherin,  $\beta$ -catenin was absent from the nucleus. An example of three cells, one expressing E-cadherin and the other two not expressing E-cadherin, is shown in Fig. 5B.  $\beta$ -catenin is in the nuclei of the cells that are not expressing E-cadherin, but it localizes to the membrane in the cell that is expressing E-cadherin.

The extracellular domain of E-cadherin is involved in cell-cell adhesion, while its intracellular domain binds  $\beta$ -catenin.  $\beta$ -catenin, in turn, binds  $\alpha$ -catenin, which connects the adherens junctions with the cytoskeleton. The ability of E-cadherin to withdraw  $\beta$ -catenin from the nucleus could come from direct binding to  $\beta$ -catenin, or indirectly from increased cell adhesion. To resolve these possibilities, we transiently transfected SW480 cells with an E-cadherin construct E $\Delta\beta\alpha$ C in which the  $\beta$ -catenin binding domain is deleted and the rest of the intracellular domain of E-cadherin is fused to the C-terminal half of  $\alpha$ -catenin. This fusion construct promotes cell adhesion although it does not bind to  $\beta$ -catenin (Nagafuchi et al., 1994). Approximately 90% of SW480 cells expressing the E $\Delta\beta\alpha$ C fusion construct maintained the nuclear localization of  $\beta$ -catenin (an example is shown in Fig. 5B), indicating that the direct binding of  $\beta$ -catenin to E-cadherin is necessary to withdraw  $\beta$ -catenin from the nucleus.



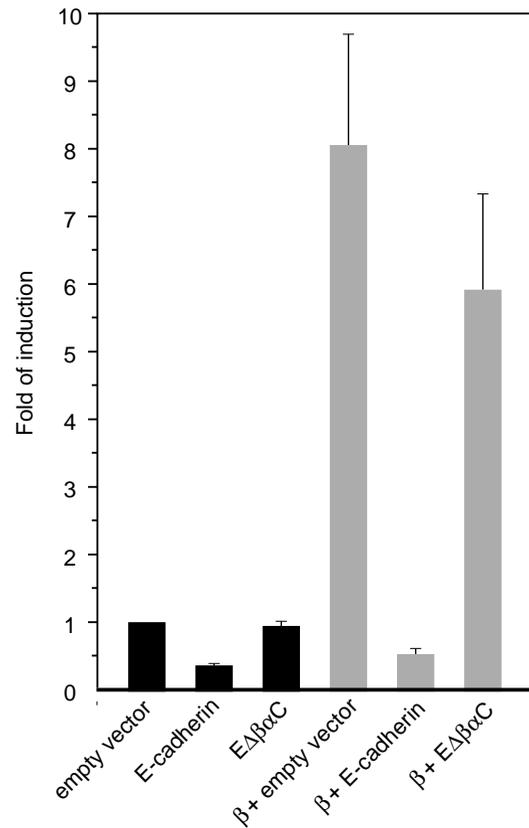
**Fig. 4.** E-cadherin $^{-/-}$  ES cells accumulate free  $\beta$ -catenin, which interacts with LEF-1 in vitro. Equivalent amounts of cell lysates of wild-type (+) and E-cadherin-negative (-) ES cells were used to assay the steady-state level of  $\beta$ -catenin in these cells (first two lanes). The steady-state level of  $\beta$ -catenin is greater in E-cadherin-expressing wild-type ES cells. The lower band is often detectable in cell lysates of ES cells and may represent a degradation product of  $\beta$ -catenin. Bands marked with an asterisk are nonspecific. The level of uncomplexed  $\beta$ -catenin in wild-type and E-cadherin $^{-/-}$  ES cells was measured by affinity precipitation with a recombinant full-length E-cadherin cytoplasmic tail (ECT.884), while a deleted version of the E-cadherin cytoplasmic domain (ECT.823) was used as a negative control. ECT.884 fusion protein precipitates free  $\beta$ -catenin only in E-cadherin $^{-/-}$  ES cells and not in wild-type ES cells. Only the free  $\beta$ -catenin in E-cadherin $^{-/-}$  ES cells interacts with LEF-1 recombinant protein. The ability of the GST-ECT recombinant proteins to precipitate uncomplexed proteins was confirmed by western blotting with an antibody against p120<sup>ctn</sup> (lower panel). IB, immunoblotting; AP, affinity precipitation.



**Fig. 5.** E-cadherin prevents nuclear localization of  $\beta$ -catenin. (A)  $\beta$ -catenin subcellular localization in wild-type, E-cadherin<sup>-/-</sup> ES cells and E-cadherin<sup>-/-</sup> ES cells re-transfected with E-cadherin under a constitutive promoter.  $\beta$ -catenin is localized to the cell membrane of wild-type ES cells (WT ES). E-cadherin<sup>-/-</sup> ES cells (E-cad<sup>-/-</sup> ES) have a nuclear/perinuclear pool of  $\beta$ -catenin but it appears to be excluded from the nucleoli (short arrow). Some cells have accumulated  $\beta$ -catenin in aggregates close to the nucleus (arrowhead). There is some membrane-associated  $\beta$ -catenin in E-cadherin<sup>-/-</sup> ES cells (long arrow) but its staining is not nearly as strong as at the membrane of E-cadherin-expressing ES cells. Re-transfection of E-cadherin<sup>-/-</sup> ES cells with E-cadherin under a constitutive promoter (E-cad<sup>-/-</sup> + E-cad ES) restores membrane localization of  $\beta$ -catenin. (B) E-cadherin sequesters nuclear  $\beta$ -catenin to the membrane. SW480 cells were transiently transfected with wild-type E-cadherin and with an E-cadherin construct that lacks the  $\beta$ -catenin binding site and which is fused with the C-terminal half of  $\alpha$ -catenin (E $\Delta\beta\alpha$ C). The E $\Delta\beta\alpha$ C construct promotes cell adhesion but does not bind to  $\beta$ -catenin. Endogenous  $\beta$ -catenin localization was visualized by immunofluorescence. SW480 cells accumulate  $\beta$ -catenin in their nuclei. In the cell that is expressing wild-type E-cadherin,  $\beta$ -catenin is present at the cell membrane but not in the nucleus. Expression of mutant E-cadherin (E $\Delta\beta\alpha$ C) does not withdraw  $\beta$ -catenin from the nucleus.

### E-cadherin prevents transactivation by $\beta$ -catenin

According to the currently accepted model, the uncomplexed  $\beta$ -catenin that accumulates in the cytoplasm enters the nucleus



**Fig. 6.** E-cadherin prevents  $\beta$ -catenin-mediated transactivation in ES cells. E-cadherin<sup>-/-</sup> ES cells were co-transfected with pCMV $\beta$ -Galactosidase as an internal standard, and with TOPFLASH as a reporter, in combination with an empty vector, wild-type E-cadherin or E-cadherin lacking the  $\beta$ -catenin-binding site (E $\Delta\beta\alpha$ C). Transcription was determined as the level of luciferase activity, which was corrected by  $\beta$ -Galactosidase activity. Numbers are given as a relative activation compared to cells transfected with an empty expression vector. Black bars represent transcriptional activity of endogenous  $\beta$ -catenin; gray bars represent transcriptional activity of endogenous and degradation-resistant S33A  $\beta$ -catenin. Expression of wild-type, but not mutant, E-cadherin reduces ectopic transactivation mediated by endogenous and overexpressed ectopic  $\beta$ -catenin.

and, in association with LEF-1/TCF, mediates transcription. Thus, the critical aspect in signaling is the increase in the level of free  $\beta$ -catenin. To test whether an excess of free  $\beta$ -catenin in E-cadherin<sup>-/-</sup> ES cells is capable of activating transcription from the LEF-1/TCF-binding promoter in E-cadherin<sup>-/-</sup> ES cells, we used the previously established  $\beta$ -catenin reporter gene assay with an optimal motif for LEF/TCF binding (van de Wetering et al., 1997). We transfected E-cadherin<sup>-/-</sup> ES cells with wild-type E-cadherin or with an E-cadherin construct that lacks the  $\beta$ -catenin binding domain to measure the effect of E-cadherin expression on the transactivation potential of endogenous  $\beta$ -catenin (Fig. 6, black bars). To measure the ability of E-cadherin to repress transcription mediated by overexpressed  $\beta$ -catenin, the cells were transfected with a degradation-resistant  $\beta$ -catenin (S33A  $\beta$ -catenin) in combination with E-cadherin constructs (Fig. 6, gray bars). Expression of wild-type E-cadherin in E-cadherin<sup>-/-</sup> ES cells reduced the transcriptional activity of endogenous  $\beta$ -catenin by

approximately threefold. On the other hand, transfection with an E-cadherin construct which does not contain the  $\beta$ -catenin binding site (E $\Delta\beta\alpha$ C) did not significantly reduce the transcriptional activity of endogenous  $\beta$ -catenin (Fig. 6, black bars). Transfection with the degradation-resistant  $\beta$ -catenin resulted in dramatically higher transcriptional activation compared to untransfected E-cadherin $^{-/-}$  ES cells. Co-transfection with wild-type E-cadherin, but not with E-cadherin that does not bind  $\beta$ -catenin, dramatically reduced the transcriptional activity of exogenous  $\beta$ -catenin (Fig. 6, gray bars). The small reduction of  $\beta$ -catenin transactivation by E $\Delta\beta\alpha$ C can be explained by a slight residual ability of this construct to attract  $\beta$ -catenin (our observation from immunoprecipitation experiments). The inhibition of  $\beta$ -catenin-mediated transactivation by wild-type, but not by mutant, E-cadherin was additionally demonstrated in SW480, NIH3T3 and 293-T cell lines. The equal expression of E-cadherin and E $\Delta\beta\alpha$ C was determined by western blotting (not shown).

## DISCUSSION

$\beta$ -catenin can play crucial and diverse roles in cadherin-mediated cell-cell adhesion, Wnt signal transduction, gene activation and tumor formation. The multiple functions of this single molecule are possible through a precise subcellular compartmentalization of  $\beta$ -catenin and the fine tuning of its concentration in each compartment. An important aspect of regulation is the ability of  $\beta$ -catenin to form mutually exclusive complexes with various binding partners present in different cell compartments. The central part of the protein consists of Arm repeats, which can bind several proteins including E-cadherin, LEF-1 and APC. The three-dimensional structure of this domain (Huber et al., 1997) reveals its tightly packed nature, and mapping studies (Behrens et al., 1996; Huber et al., 1996b; Hulsken et al., 1994b; Orsulic and Peifer, 1996) show that the binding sites for E-cadherin, LEF-1 and APC are partially overlapping. It was previously shown that APC and cadherin form independent and competitive complexes with  $\beta$ -catenin (Hulsken et al., 1994b; Rubinfeld et al., 1995). We have shown here that LEF-1 and E-cadherin form mutually exclusive complexes with  $\beta$ -catenin, as do LEF-1 and APC. The competitive nature of these complexes would ensure that transcriptionally active  $\beta$ -catenin is not subject to degradation or membrane function at the same time, thus functioning as a molecular switch.

A subtle change in the level of signaling-competent  $\beta$ -catenin may have a drastic impact on gene activation. It was shown, for example, that relatively small differences in free  $\beta$ -catenin on the dorsal, versus ventral, side of the *Xenopus* embryo resulted in axis formation (Larabell et al., 1997). The idea that Wnt signaling and cadherins compete for the same pool of  $\beta$ -catenin is primarily based on work with *Xenopus* and *Drosophila*. In *Xenopus*, expression of various Wnt genes in ventral blastomeres results in dorsalization and axis duplication (McMahon and Moon, 1989). The same effect is obtained by overexpression of ectopic  $\beta$ -catenin (Funayama et al., 1995). Injection of high levels of ectopic cadherin inhibits dorsal axis formation, suggesting that cadherins compete with Wnts and deplete the signaling-competent pool of  $\beta$ -catenin

(Fagotto et al., 1996; Heasman et al., 1994; Torres et al., 1996). Consistent with this interpretation, *armadillo*-mutant *Drosophila* embryos with half of the normal amount of cadherin have a less severe segment polarity phenotype than *armadillo*-mutant embryos with wild-type cadherin expression. The reduction of cadherin evidently frees up some of the maternal storage of wild-type Armadillo protein from the adherens junctions, which then can function in Wingless signaling (Cox et al., 1996). Cadherin overexpression, on the other hand, mimics the *wingless* phenotype in *Drosophila* (Sanson et al., 1996).

In a simple model, E-cadherin stabilizes  $\beta$ -catenin at the membrane and sequesters  $\beta$ -catenin that has escaped the degradation mechanism in the cytoplasm. The Wnt signal inhibits the degradation machinery in the cytoplasm and overwhelms the binding capacity of E-cadherin, resulting in an excess of free  $\beta$ -catenin, which then enters the nucleus and activates its target genes. We have shown that the loss of E-cadherin expression in ES cells permits free  $\beta$ -catenin to accumulate and associate with the LEF-1 transcription factor. In this sense, loss of membrane E-cadherin mimics Wnt signaling. Consistent with this model, we have demonstrated that E-cadherin $^{-/-}$  ES cells accumulate free  $\beta$ -catenin in the cell nuclei and that the overexpression of E-cadherin can result in withdrawal of  $\beta$ -catenin from the nucleus and sequestering it at the membrane. We further showed that  $\beta$ -catenin transcriptional function is antagonized by E-cadherin expression in E-cadherin $^{-/-}$  ES cells. The inhibitory effect of E-cadherin on  $\beta$ -catenin transcription is not mediated by increased cell adhesion but by direct binding to  $\beta$ -catenin. Recently, two other partners of  $\beta$ -catenin, N-cadherin and  $\alpha$ -catenin, which are active in cell adhesion, were shown to antagonize the nuclear localization of  $\beta$ -catenin and its function in transcription (Sadot et al., 1998; Simcha et al., 1998).

The Wnt-1 proto-oncogene promotes tumor progression in mice and also upregulates  $\beta$ -catenin levels in mammalian cells (Papkoff et al., 1996; Tsukamoto et al., 1988). Overexpression of a mutant  $\beta$ -catenin lacking the N-terminal domain transforms NIH 3T3 cells (Whitehead et al., 1995). High levels of cytoplasmic and nuclear  $\beta$ -catenin have been detected in tumor tissue sections as well as in colon carcinoma (Inomata et al., 1996; Korinek et al., 1997; Morin et al., 1997; Munemitsu et al., 1995) and melanoma cell lines (Robbins et al., 1996; Rubinfeld et al., 1997). These authors speculated about a prolonged half-life of  $\beta$ -catenin and formation of a constitutive transcriptional complex, which would contribute to inappropriate gene activation as the basis for transformation of these cells. If the critical step in activating LEF-1/ $\beta$ -catenin-driven transcription is merely an increase in free  $\beta$ -catenin, E-cadherin should have an important role as a negative regulator of  $\beta$ -catenin signaling. Indeed, 50-80% of highly metastatic carcinomas downregulate expression or function of E-cadherin or  $\alpha$ -catenin (reviewed in Birchmeier and Behrens, 1994); re-establishing the function of E-cadherin reverts tumor cell lines to a benign epithelial phenotype (Vleminckx et al., 1991) and arrests tumor development (Perl et al., 1998). Thus, in cancers which downregulate E-cadherin, an increase in free  $\beta$ -catenin may be the mechanism for their transformation.

The finding that  $\beta$ -catenin interacts with LEF/TCF nuclear factors (Behrens et al., 1996; Huber et al., 1996b; Molenaar et

al., 1996) suggests how  $\beta$ -catenin could activate transcription. E-cadherin could potentially antagonize signaling activity of  $\beta$ -catenin by sequestering  $\beta$ -catenin at the membrane and thus keeping it away from the nucleus, as well as by competing with LEF-1 for the same binding sites on  $\beta$ -catenin. In E-cadherin $^{-/-}$  ES cells, LEF-1 expression is upregulated (Huber et al., 1996a) and free  $\beta$ -catenin accumulates. The abundant  $\beta$ -catenin/LEF-1 complex here is probably the critical component for transcriptional activation.

The nature of the target genes that would contribute to cell transformation is not yet known. Recently, we have performed a differential display of the mRNA populations of wild-type versus E-cadherin $^{-/-}$  ES cells. It is striking that most of the differentially expressed genes have previously been identified as being upregulated in different epithelial cancers (S. Orsulic and R. Kemler, unpublished). Interestingly, in one model (Huber et al., 1996a), E-cadherin itself is a target of  $\beta$ -catenin and its transcription is downregulated upon binding of the  $\beta$ -catenin/LEF-1 complex to the E-cadherin promoter. This mechanism would lead to an irreversible cycle of accumulation of free  $\beta$ -catenin and cell transformation through inappropriate activation of other target genes.

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