ITF-2, a downstream target of the Wnt/TCF pathway, is activated in human cancers with β-catenin defects and promotes neoplastic transformation

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Summary

In many cancers, inactivation of the adenomatous polyposis coli (APC) or Axin tumor suppressor proteins or activating mutations in β-catenin lead to elevated β-catenin levels, enhanced binding of β-catenin to T cell factor (TCF) proteins, and increased expression of TCF-regulated genes. We found that the gene for the basic helix-loop-helix transcription factor ITF-2 (immunoglobulin transcription factor-2) was activated in rat E1A-immortalized RK3E cells following neoplastic transformation by β-catenin or ligand-induced activation of a β-catenin-estrogen receptor fusion protein. Human cancers with β-catenin regulatory defects had elevated *ITF-2* expression, and *ITF-2* was repressed by restoring wild-type APC function or inhibiting TCF activity. Of note, *ITF-2* promoted neoplastic transformation of RK3E cells. We propose that *ITF-2* is a TCF-regulated gene, which functions in concert with other TCF target genes to promote growth and/or survival of cancer cells with defects in β-catenin regulation.

Introduction

Wnt signaling plays a central role in regulating proliferation, differentiation, and morphogenesis, and control of β-catenin stability is pivotal in Wnt signaling (reviewed in Cadigan and Nusse, 1997; Peifer and Polakis, 2000; Bienz and Clevers, 2000; Polakis, 2001). In brief, Wnt ligands activate transmembrane frizzled receptors, leading to inhibition of glycogen synthase kinase-3β (GSK-3β) activity. The GSK-3β protein, when active and complexed with the APC (adenomatous polyposis coli) and Axin proteins, can phosphorylate specific serine and/or threonine residues near β-catenin's amino (N) terminus (Zeng et al., 1997; Ikeda et al., 1998; Behrens et al., 1998; Yamamoto et al., 1998; Polakis, 2001). The phosphorylated forms of β-catenin bind the F box protein β-TrCP, a subunit of the SCF-type E3 ubiquitin ligase complex, resulting in ubiquitination of β-catenin and its destruction by the proteasome (Jiang and Struhl, 1998; Winston et al., 1999; Hart et al., 1999; Kitagawa et al., 1999; Polakis, 2001). Following Wnt pathway activation and inhibition of GSK-3β, β-catenin accumulates in the cytoplasm and nucleus, where it can bind to members of the TCF (T cell factor)/LEF (lymphoid enhancer family) transcription factor family (Behrens et al., 1996; Molenaar et al., 1996) (referred to here collectively as TCFs). In the nucleus, TCFs mediate sequence-specific DNA binding, and β-catenin, via interaction with TCFs, appears to have major effects on transcription of genes with TCF binding sites in their regulatory regions (reviewed in Peifer and Polakis, 2000; Bienz and Clevers, 2000; Polakis, 2000).

Defects in Wnt signaling play a major role in human cancer. 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 many different cancer

SIGNIFICANCE

A number of TCF-regulated genes in cancer have been suggested. A fraction of the genes, such as *c-MYC* and *cyclin D1*, have been well established to function as oncogenes when deregulated. A few others, such as *MMP-7* and *gastrin*, have been implicated in the genesis of mouse tumors arising from β-catenin deregulation. However, most suggested TCF-target genes have not been shown to have critical functions in neoplastic transformation in model systems or human cancer. Our findings establish *ITF-2* as a TCF target gene capable of promoting neoplastic transformation of epithelial cells in a fashion akin to mutant β-catenin. Moreover, our data showing that elevated *ITF2* expression is linked to mutational defects in β-catenin regulation in primary ovarian carcinomas implicate *ITF-2* in human cancer.

types (Polakis, 2000). In upwards of 70%–80% of colorectal cancers and a fraction of other cancers, inactivation of the *APC* tumor suppressor gene is the predominant mechanism leading to β-catenin deregulation (Kinzler and Vogelstein, 1996; Bienz and Clevers, 2000). In other cancers, mutations in the genes encoding one of the two Axin proteins have been reported (Satoh et al., 2000; Liu et al., 2000; Dahmen et al., 2001; Wu et al., 2001). A presumed critical consequence of the Wnt pathway mutations, whether in the *CTNNB1*, *APC*, or *AXIN* genes, is elevation of β-catenin levels in the cytoplasm and nucleus. Deregulation of β-catenin leads to constitutive formation of β-catenin-TCF complexes and altered expression of TCF target genes. Proteins encoded by TCF-regulated genes likely cooperate in effecting neoplastic transformation. Proposed Wnt/TCF target genes in cancer cells include known oncogenes, such as *c-MYC* (He et al., 1998) and *CCND1* (*Cyclin D1*) (Tetsu and McCormick, 1999; Shtutman et al., 1999), as well as *MMP-7* (*matrix metalloproteinase 7/matrilysin*) (Crawford et al., 1999), *TCF7* (Roose et al., 1999), *PPAR-δ* (*peroxisome proliferator-activated receptor delta*) (He et al., 1999), *WISP-1* (Xu et al., 2000), *gastrin* (Koh et al., 2000), *MDR1* (Yamada et al., 2000), *AXIN2* (Yan et al., 2001), and *survivin* (Zhang et al., 2001).

In an effort to better understand the contribution of Wnt/β-catenin/TCF pathway defects in cancer, we have pursued efforts to identify potential downstream target genes in RK3E cells, a rat E1A-immortalized epithelial cell line that can be neoplastically transformed by mutant β-catenin (Kolligs et al., 1999). Studies in *Drosophila* have implicated the Wnt pathway in regulating the proneural *acheate-scute* genes (Couso et al., 1994; González-Gaitán and Jäckle, 1995; Johnston and Edgar, 1998; Wan et al., 2000), which encode basic helix-loop-helix (bHLH) transcription factors. In Northern blot studies in RK3E cells, we failed to find evidence that mammalian homologs of *acheate-scute* were regulated by β-catenin. Because of their role in regulating the activity of acheate-scute-type bHLH proteins, we pursued pilot studies of the expression of genes encoding class I or "E type" bHLH proteins. The four class I bHLH proteins play important roles in cell growth and differentiation. They are characterized by a broad expression pattern and the capability of forming homodimers as well as heterodimers with other classes of bHLH proteins (reviewed in Murre et al., 1994; Atchley and Fitch, 1997; Massari and Murre, 2000). Following dimerization, the bHLH proteins can recognize a consensus DNA sequence termed an E box (i.e., 5′-CANNTG-3′). Dimerization is mediated via the HLH domain, whereas DNA binding is conferred by the basic region. In contrast to class I bHLH proteins, class II bHLH proteins typically show tissue-specific expression patterns and a stronger potential for forming heterodimers with E proteins than for forming homodimers (Murre et al., 1994; Massari and Murre, 2000). A related class of proteins, commonly named Ids, lacks the DNA binding domain but still efficiently dimerizes with E proteins via the HLH motif (reviewed in Massari and Murre, 2000; Norton, 2000). The Ids are presumed to negatively regulate differentiation pathways by sequestering E proteins, thereby inhibiting formation of heterodimers of E proteins and tissue specific bHLH-proteins.

Intriguingly, we found that the gene encoding the class I bHLH protein ITF-2 (immunoglobulin transcription factor) (also known as E2-2, SEF-1 and Tcf-4 [for transcription factor 4]; hereafter referred to as ITF-2) showed reproducibly and significantly increased expression in RK3E cells following β-catenin-induced neoplastic transformation. In addition to its activation in RK3E cell lines stably transformed by mutant β-catenin, *ITF-2* was strongly activated in RK3E cells stably transformed by γ-catenin or following ligand-induced activation of a β-catenin-estrogen receptor (ER) fusion protein. Further studies implicated TCFs and β-catenin in regulation of *ITF-2* expression in human cancers. Consistent with the notion that *ITF-2* deregulation plays a vital role in cancers with β-catenin defects, aberrant expression of *ITF-2* in RK3E cells promoted neoplastic transformation.

Results

Activation of *ITF-2* expression by β- and γ-catenin in RK3E cells

Our prior studies have shown that N-terminal mutant forms of β-catenin akin to those found in cancers, but not wild-type β-catenin, promote neoplastic transformation of RK3E cells (Kolligs et al., 1999). Unlike β-catenin, its close functional relative γ-catenin (also known as plakoglobin) will promote neoplastic transformation of RK3E cells when overexpressed, without a need for N-terminal mutations in the presumptive GSK-3β phosphorylation consensus sites to activate γ-catenin's transforming potential (Kolligs et al., 2000). As noted above, while pilot Northern blot studies failed to show that mammalian homologs of *acheate-scute* genes were regulated by β-catenin in RK3E cells, we did find that the gene encoding the class I bHLH protein ITF-2 was activated in β-catenin-transformed RK3E cell lines. To establish the significance of this observation, additional Northern blot studies of *ITF-2* expression were carried out on RNA from a larger panel of cell lines, including parental RK3E cells, nine independent β-catenin-transformed RK3E lines, and nine independent γ-catenin transformed lines. *ITF-2* expression was markedly and consistently increased in β- and γ-catenin transformed RK3E lines, ranging from 2.4- to 11.6-fold greater than the levels seen in parental RK3E cells (Figure 1A). The observation that *ITF-2* expression was not increased in RK3E cells transformed by mutant K-ras (RK3E/K-ras) suggested that increased expression in β- and γ-catenin-transformed lines did not simply reflect their neoplastic phenotype. An analysis of the time course of *ITF-2* induction following infection of RK3E cells with retroviruses encoding the S33Y oncogenic form of β-catenin or a control β-galactosidase protein showed that *ITF-2* expression was induced roughly concordant with accumulation of exogenous β-catenin protein in the cells (Figure 1B and data not shown). Consistent with the view that β-catenin activates *ITF-2* expression via TCF-dependent mechanisms, induction of *ITF-2* by β-catenin was clearly inhibited in an RK3E cell line stably expressing a dominant negative mutant form of TCF-4 (i.e, RK3E/Tcf-4ΔN31 cells; Figure 1B).

To confirm the link between activation of β-catenin and induction of *ITF-2* expression, we utilized an RK3E cell line expressing a chimeric β-catenin-estrogen receptor (ER) fusion protein (RK3E/S33Y-ER), in which full-length S33Y β-catenin sequences had been cloned upstream of a mutated estrogen receptor ligand binding domain capable of binding 4-hydroxytamoxifen (4-OHT) but not estrogen (Littlewood et al., 1995). Following treatment of the RK3E/S33Y-ER cell line with 4-OHT, gel shift and reporter gene assays indicated that β-catenin/TCF complexes and elevated TCF transcriptional activity could first be detected between 6–12 hr (Figure 2C and data not shown). *ITF-2* transcripts were increased roughly 1.5-fold over control

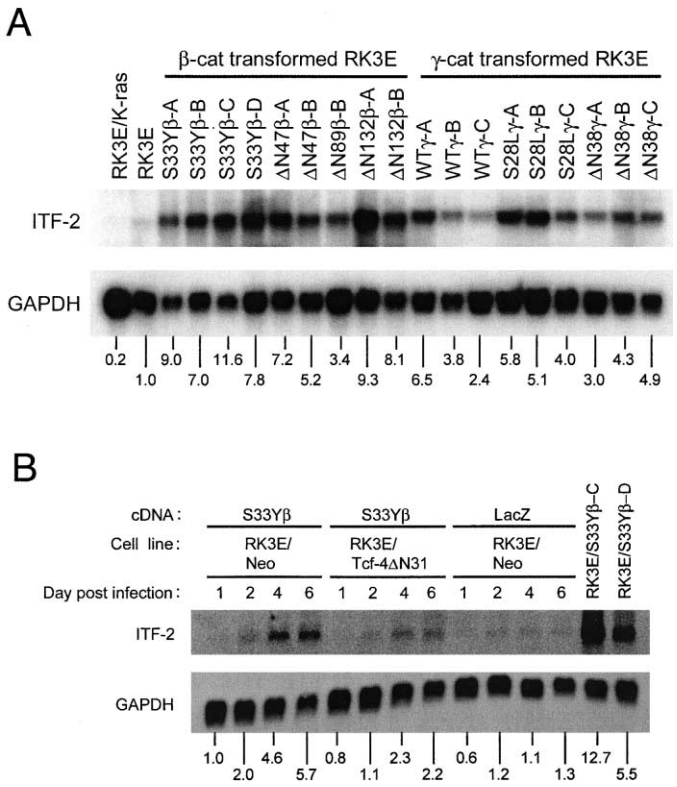

Figure 1. Activation of *ITF-2* expression by β- and γ-catenin in RK3E cells

A: Northern blot studies of *ITF-2* expression were carried out on RNA from parental RK3E cells, a polyclonal RK3E line transformed by mutant K-ras (RK3E/K-ras), and 18 independent clonal RK3E cell lines stably transformed by β- or γ-catenin. **B:** Northern blot studies of *ITF-2* expression at various time points following infection of RK3E/Neo and RK3E/Tcf-4ΔN31 cells with retroviruses containing the indicated cDNAs for S33Y β-catenin and LacZ (β-galactosidase). RNA from clonal RK3E lines stably transformed by β-catenin (RK3E/S33Yβ-C and RK3E/S33Yβ-D) are shown at the right. To assess loading and transfer, the blots were stripped and hybridized to a *GAPDH* probe. The levels of *ITF-2* expression in each cell line, after normalization for the *GAPDH* signal, are indicated below the respective lanes, with values relative to those in parental RK3E cells (**A**) or day 1 post-infection of S33Yβ-catenin infected RK3E/Neo cells (**B**). The largest and most intense band detected on the *ITF-2* Northern blots, corresponding to *ITF-2B* isoform transcripts, was quantified and is shown in the figure.

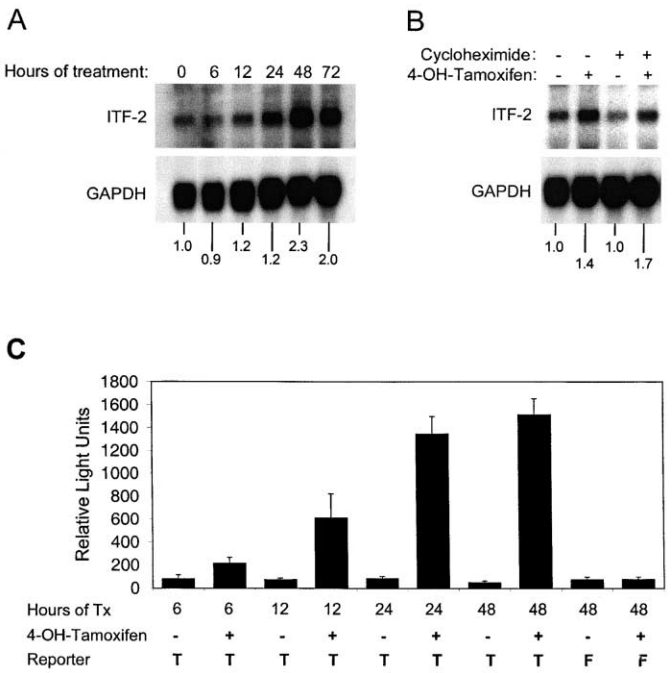

Figure 2. *ITF-2* is a direct target of β-catenin in RK3E cells

A: Time course of *ITF-2* induction in the RK3E/S33Y-ER cell line following exposure to 4-OHT to activate the S33Y β-catenin/ER chimeric protein. Northern blot analysis was performed on total RNA isolated at the indicated time points. **B:** Induction of *ITF-2* following β-catenin activation by 4-OHT treatment does not require new protein synthesis. Northern blot analysis of *ITF-2* expression in RK3E/S33Y-ER cells in control conditions or following a 12 hr exposure to 4-OHT and/or cycloheximide. Following hybridization to the *ITF-2* probe, the blots were stripped and rehybridized to a *GAPDH* probe. The level of ITF-2 expression in each sample, after normalization for the *GAPDH* signal, is indicated below the respective lane. The largest and most intense band detected on the *ITF-2* Northern blots, corresponding to *ITF-2B* isoform transcripts, was quantified and is shown in the figure. **C:** Time course of TCF reporter gene activation in the RK3E/S33Y-ER cell line. 24 hr after transfection with the pTOPFLASH (T) or pFOPFLASH (F) reporter gene constructs, cells were either sham treated (−) or exposed to 4-OHT (+) for the indicated time periods, prior to harvesting the cells for luciferase assays. The means and standard deviations of light units detected in two independent experiments performed in duplicate are shown.

levels after 12 hr of 4-OHT treatment to more than 2-fold over control levels during the time course studied (Figures 2A and 2B and data not shown). Consistent with the notion that *ITF-2* is a "direct" or primary target gene regulated by β-catenin, blockade of new protein synthesis by cycloheximide treatment did not inhibit the induction of *ITF-2* at the 12 hr time point (Figure 2B). More extended treatment of the cells with cycloheximide resulted in cell death. The induction of *ITF-2* transcripts following 4-OHT treatment may be perceived as delayed relative to that expected for a direct target gene. However, because TCF activity is not strongly activated in the RK3E/S33Y-ER cell line until about 12 hr after 4-OHT treatment, and the *ITF-2* transcriptional unit is very large (>350 kilobases; see below), requiring more than 3 hr to transcribe, these factors may contribute to the apparent delay in *ITF-2* transcript accumulation after 4-OHT treatment.

Repression of *ITF-2* expression by APC and dominant negative TCF-4

As noted above, inactivating mutations in the *APC* gene are seen in about 70%–80% of human colon cancers, and the defects lead to β-catenin deregulation and constitutive activation of β-catenin/TCF transcripton (Kinzler and Vogelstein, 1996; Bienz and Clevers, 2000). To explore the relationship between β-catenin deregulation and *ITF-2* expression in human colon cancer, we took advantage of a colon cancer cell line with tightly regulated expression of an exogenous copy of the wild-type *APC* gene. The HT29 colon cancer line has truncating mutations in both *APC* alleles, and a variant HT29 line (HT29/APC) has been generated in which, following zinc treatment, expression of an exogenous wild-type APC protein is rapidly induced to roughly the same level as that of the endogenous truncated APC proteins (Morin et al., 1995). Using real-time RT-PCR assays to study gene expression at various time points following zinc treatment of the HT29/APC cells and a matched control line

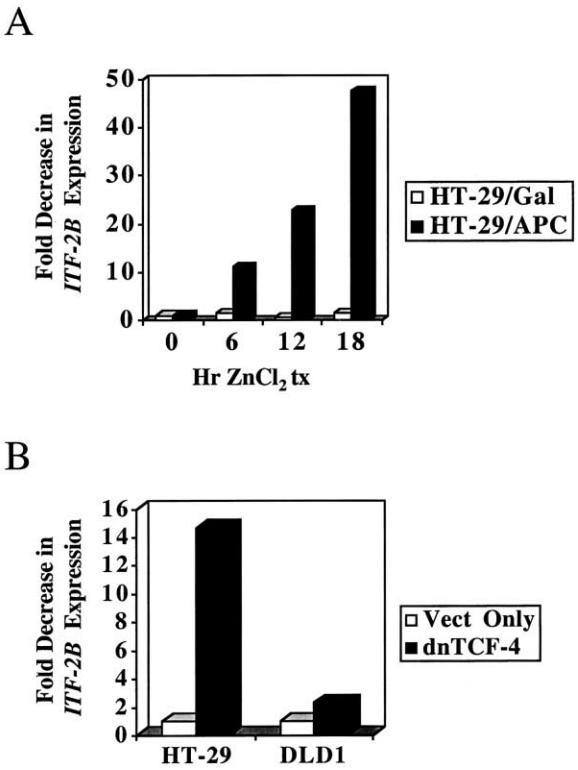

Figure 3. Repression of *ITF-2B* expression in colon cancer cells by APC and dominant negative TCF-4

A: Quantitative real-time (TaqMan) RT-PCR analysis of *ITF-2B* expression at various time points following $ZnCl_2$ treatment (tx) of HT-29 cells with Zn-inducible APC expression (HT-29/APC) or HT-29 cells with Zn-inducible β-galactosidase (HT-29/Gal). Following normalization for *HPRT* expression, the data are plotted as the fold decrease in *ITF-2B* expression relative to the control time point (0 hr). **B:** Real-time RT-PCR analysis of *ITF-2B* expression in HT-29 and DLD1 colon cancer cells stably expressing a dominant negative form of TCF-4 (dnTCF-4) or control empty expression vector cassette (vector only). Following normalization for *HPRT* expression, data are plotted as the fold decrease in *ITF-2B* expression, with the control (vector only) assigned a value of 1.

(i.e., HT29/β-Gal), we found that *ITF-2* expression was strongly suppressed by APC induction (Figure 3A). Zinc treatment of the control HT29/β-gal cell line had no detectable effect on *ITF-2* expression (Figure 3A). TCFs seem to play an important role in β-catenin's ability to regulate *ITF-2* expression in colon cancer, as *ITF-2* expression was reduced in both HT-29 and DLD-1 colon cancer cells by expression of a dominant negative form of TCF-4 (dnTCF-4), in which the N-terminal β-catenin binding region of TCF4 had been deleted (Figure 3B).

β-catenin activates *ITF-2B* transcription via a TCF site in the proximal promoter

Based on sequence analysis, the human *ITF-2* gene maps to chromosome 18q21, and transcripts encoding multiple isoforms of the protein have been described (Henthorn et al., 1990; Corneliussen et al., 1991; Skerjancet al., 1996; Liu et al., 1998). Two of the major isoforms, ITF-2A and ITF-2B, differ at their N termini, but share identity over the majority of their extent, including the bHLH region. Specifically, the predicted 667 amino acid ITF-2B isoform shares the carboxyl-terminal 483 amino acids with the predicted 510 amino acid ITF-2A isoform. Based on examination of the human genomic DNA sequence, the *ITF-2B* transcriptional unit spans greater than 350 kilobases. Transcripts for the two isoforms appear to arise as the result of alternative promoter usage, with the 5′-most exon for the shorter *ITF-2A* isoform located within what appears to be intron 7 of the transcriptional unit for the longer *ITF-2B* isoform. Our real-time RT-PCR studies in the HT-29 and DLD-1 colon cancer cell lines indicated that restoration of APC function or expression of dnTCF-4 significantly reduced *ITF-2B* transcript levels, but had very modest effects on the levels of *ITF-2A* transcripts (Figure 3 and data not shown). Although the start site for human *ITF-2B* transcripts has not been mapped, a search of the genomic sequences flanking exon 1 of the *ITF-2B* transcriptional unit identified several potential TCF binding sites upstream of exon 1 (Figure 4A). Cloning of rat genomic *ITF-2* sequences and analysis of the 5′ extent of *ITF-2B* transcripts in β-catenin-transformed RK3E cells revealed the presence of potential TCF binding sites upstream of rat *ITF-2B* exon 1.

Luciferase reporter gene constructs containing sequences from the presumptive human and rat *ITF-2B* promoters were generated, along with analogous constructs containing mutations in proximal potential TCF binding sites. We found that β-catenin could stimulate luciferase activity of the rat reporter gene constructs containing wild-type TCF elements (i.e., constructs rITF2α and rTF2β; Figure 4B), but not constructs with deletion or mutation of a proximal TCF binding site (i.e., constructs rITF2γ and rITF2δ; Figure 4B). Similar to the findings obtained in studies of other candidate β-catenin/TCF regulated genes, such as *c-MYC* (He et al., 1998), *CCND1* (Tetsu and McCormick, 1999), and *MMP-7* (Crawford et al., 1999), the rat *ITF-2B* promoter was only modestly responsive to β-catenin in cells with otherwise intact β-catenin/TCF function (i.e., 293 cells). Therefore, we sought to obtain additional evidence supporting the role of β-catenin and TCFs in regulating the *ITF-2B* promoter. As such, the transcriptional activity of the human *ITF-2B* 5′ flanking region was assessed in colon cancer cell lines that had deregulated TCF transcription resulting from an oncogenic mutation in β-catenin (HCT-116) or APC inactivation (SW480) (Morin et al., 1997). The wild-type human *ITF-2B* reporter construct (i.e., hITF2α) had strong activity in HCT-116 and SW480 cells relative to the TCF-dependent TOPFLASH reporter construct, and this activity could be significantly inhibited by cotransfection of the dnTCF-4 expression construct (Figure 4C). The activity of the hITF2β reporter construct containing mutations in a proximal TCF binding element was substantially reduced compared to the ITF2α construct in both HCT-116 and SW480 cells, and its activity was nominally affected by cotransfection of the dnTCF-4 construct (Figure 4C). Taken together, the data from the studies of the rat and human *ITF-2B* promoters suggest that proximal TCF binding elements play a key role in β-catenin's ability to activate *ITF-2B* expression in cancer cells.

Increased *ITF-2B* expression in cancers with β-catenin defects

Many of the candidate β-catenin/TCF-regulated genes described in the literature have been proposed based largely on data from in vitro and/or animal model studies. To date, only a few studies have assessed expression of candidate β-catenin/TCF target genes in collections of primary human tumors that have been thoroughly characterized for mutational defects in

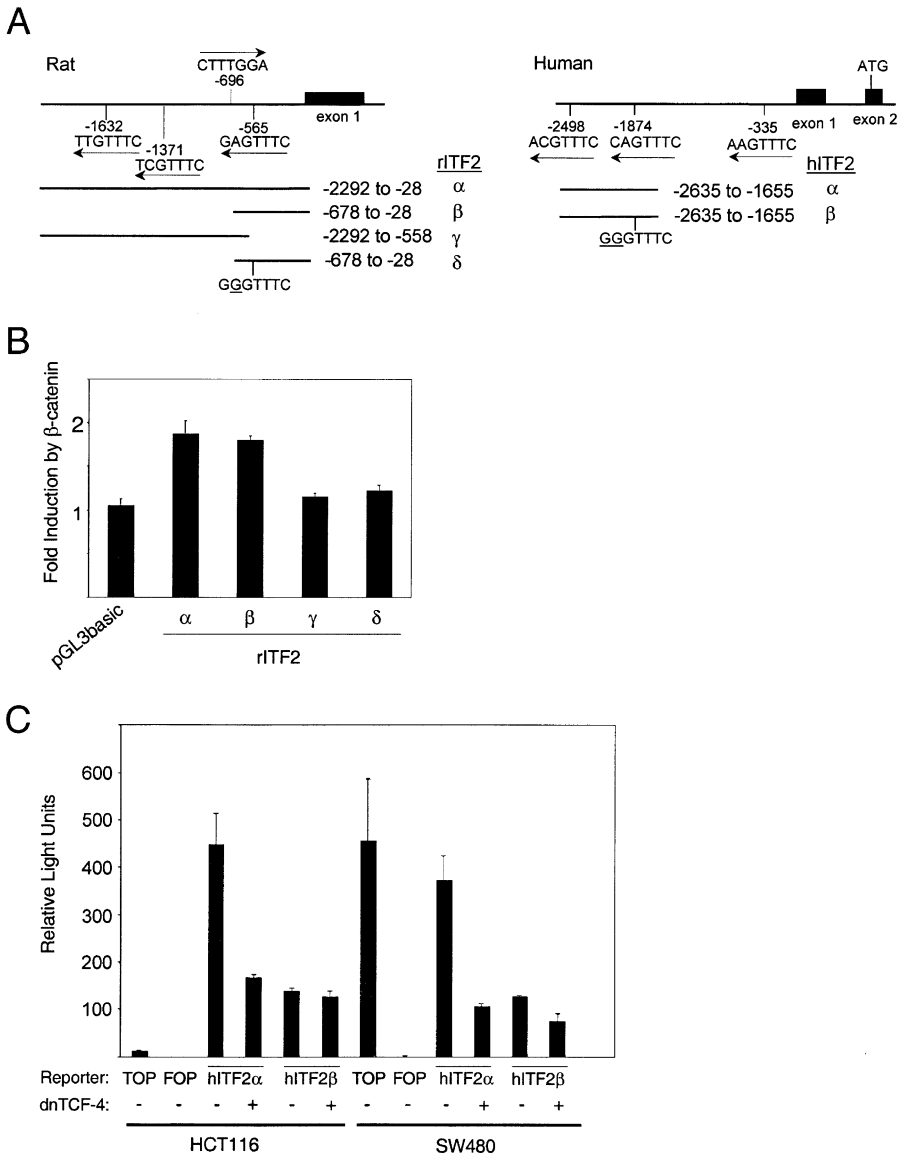

Figure 4. Role of TCF elements in the rat and human *ITF-2B* promoters in regulating transcriptional activity

A: Schematic diagram of rat and human *ITF-2B* promoter regions, indicating location of 5′ exons, candidate TCF binding sites, and the extent of the rat and human *ITF-2* sequences cloned into the pGL3basic luciferase reporter vector. The designation for the 4 rat and 2 human ITF2 reporter constructs and the status (wild-type or mutated) of candidate TCF binding sites in the constructs are noted. The transcriptional initiation sites for rat and human *ITF-2B* have not been definitively mapped. Position −1 in the rat *ITF-2B* promoter was designated as the nucleotide immediately upstream of the 5′ end of a rat 5′ RACE product. For the human *ITF-2B* promoter, −1 was designated as the nucleotide immediately upstream of the 5′-most nucleotide present in the human *ITF-2B* cDNA sequence in the GenBank accession. However, based on the activity of the hITF2α reporter construct, promoter elements and transcriptional initiation appear to be considerably upstream of −1. The direction of the arrows indicates the strand on which the candidate TCF binding elements were detected. The underlined nucleotide(s) indicate the mutations present in the mutated rat and human reported constructs. **B:** Effects of β-catenin on rat ITF2 reporter gene constructs in 293 cells. The relative activity of control pGL3basic and rat ITF2 constructs shown in **A** was assessed following transient transfection of the cells with a pcDNA3 expression construct encoding S33Y β-catenin. The fold induction by β-catenin relative to cotransfection with empty pcDNA3 vector is shown. The means and standard deviations of three independent experiments are shown. **C:** Activity of human ITF2 reporter constructs and their response to dominant negative TCF-4 (dnTCF-4) in human HCT116 and SW480 colon cancer cells. The reporter gene transfected is indicated along with whether dnTCF-4 was cotransfected. In all assays, the amount of DNA transfected was kept constant by cotransfection of empty vector, if required. The means and standard deviations of the light units detected in two independent experiments performed in duplicated are shown.

β-catenin regulation (Takayasu et al., 2001; Yan et al., 2001). We examined *ITF-2* expression in primary ovarian endometrioid adenocarcinomas (OEAs), because, while OEAs share very comparable histological features, only about 30%–40% of the lesions have mutational defects affecting β-catenin regulation (Gamallo et al., 1999; Saegusa and Okayasu, 2001; Wu et al., 2001). This contrasts with the situation in primary colorectal carcinomas, which nearly always carry mutational defects in β-catenin regulation (Kinzler and Vogelstein, 1996; Bienz and Clevers, 2000). As such, comparison of gene expression in OEAs with intact β-catenin regulation versus OEAs with defective β-catenin regulation should permit a more definitive evaluation to be made regarding the relationship between β-catenin regulatory defects and expression of candidate β-catenin/TCF target genes. Using real-time RT-PCR assays to assess *ITF-2A* and *ITF-2B* expression in a group of 45 OEAs previously characterized for β-catenin nuclear localization and mutations in the β-*catenin*, *APC*, *AXIN1*, and *AXIN2* genes (Wu et al., 2001 and data not shown), we found a statistically significant increase in the levels of *ITF-2A* and *ITF-2B* transcripts when comparing OEAs with β-catenin regulatory defects to OEAs with intact β-catenin regulation ($P = 0.048$ for *ITF-2A* and $P = 0.0026$ for *ITF-2B*). In addition to the fact that the data showed greater statistical significance for *ITF-2B*, the magnitude of the increase in *ITF-2B* transcripts in tumors with β-catenin defects was far greater than that observed for *ITF-2A* (Figure 5).

Immunohistochemistry studies with polyclonal antibodies raised against sequences shared by both ITF-2A and ITF-2B were carried out on a subset of the OEAs studied in the real-time RT-PCR analysis. In six of eight OEAs with β-catenin regulatory defects and elevated *ITF-2B* gene expression, moderate to strong reactivity for ITF-2 was seen in the nuclei of the carcinoma cells (examples shown in Figures 6B–6E). Studies of adjacent sections from selected cases using antibodies against β-catenin and ITF-2 demonstrated concordant nuclear staining patterns for the two antibodies (Figures 6A and 6B), though, as found

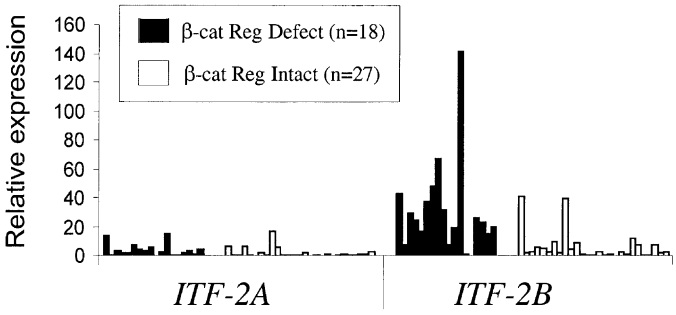

Figure 5. *ITF-2B* expression is markedly increased in ovarian endometrioid adenocarcinomas (OEAs) with β-catenin regulation defects

cDNA preparations from snap-frozen OEA specimens that had been previously studied for β-catenin immunohistochemistry ,and mutations in critical Wnt pathway components (β-catenin, APC, AXIN1, and AXIN2) were subjected to quantitative real-time (TaqMan) analysis of *ITF-2A* and *ITF-2B* expression. Following normalization for *HPRT* expression, data are plotted showing the relative levels of *ITF-2A* and *ITF-2B* expression in the 18 tumor samples with strong nuclear staining for β-catenin and mutations in the β-*catenin*, *APC*, *AXIN1*, or *AXIN2* genes (i.e., β-catenin Reg [Regulation] Defect) versus the levels of *ITF-2A* and *ITF-2B* expression in the 27 OEAs lacking strong nuclear β-catenin staining and pathway mutations (i.e., β-catenin Reg Intact).

previously (Wu et al., 2001), the β-catenin antibody often showed strong cytoplasmic staining along with nuclear staining (Figure 6A). Six of the seven OEAs with intact β-catenin regulation showed virtual absence of ITF-2 immunoreactivity in the nuclei of carcinoma cells, while the remaining case showed weak nuclear staining for ITF-2 (examples in Figure 6F–6H). The immunohistochemistry results offer further support for the notion that defects in β-catenin regulation lead to significant alterations in ITF-2 expression in primary human cancers.

ITF-2B promotes neoplastic transformation

As described above, cancer-derived mutant forms of β-catenin, but not wild-type β-catenin, will readily generate foci of morphologically transformed cells when introduced into the RK3E E1A-immortalized rat epithelial cell line, and the ability of β-catenin to induce neoplastic transformation in RK3E is dependent on intact TCF function (Kolligs et al., 1999). In our studies, several candidate β-catenin/TCF target genes, including *c-MYC* and *WISP-1*, have not shown focus-forming activity in RK3E cells (F.T.K. and E.R.F., unpublished data), though the result for *c-MYC* may be attributable to the fact that the presence of E1A in RK3E cells renders *c-MYC* redundant. Nevertheless, given the utility of the RK3E system for assessing the transforming activity of β-catenin and the evidence that *ITF-2B* is a β-catenin/TCF-regulated target gene, we sought to determine if *ITF-2B* had focus-forming activity in RK3E. Consistent with our prior studies (Kolligs et al., 1999, 2000), replication-defective retroviruses expressing the control *LacZ* gene failed to induce foci in RK3E cells, and retroviruses expressing the S33Y mutant form of β-catenin induced more than 300 foci (Figures 7A, 7B, and 7E). Remarkably, replication-deficient retroviruses expressing *ITF-2B* generated foci of morphologically transformed cells in RK3E, albeit with an efficiency roughly one-third that of S33Y β-catenin retroviruses (Figures 7C and 7E). Unlike β-catenin and γ-catenin, which are dependent on intact TCF function to induce morphological transformation of RK3E cells (Kolligs et al., 1999, 2000), focus formation by *ITF-2B* was not detectably inhibited in RK3E cells that stably overexpressed the dnTCF-4 mutant protein (Figures 7D and 7E). While parental RK3E cells are incapable of forming colonies in soft agar (i.e., anchorage-independent growth), 13 of 21 independent clonal cell lines established from ITF-2B transformed RK3E foci showed robust growth in soft agar, with colony forming abilities in agar akin to those seen in β-catenin-tranformed RK3E lines (data not shown). Taken together, the data establish that *ITF-2B* can induce neoplastic transformation in epithelial cells when deregulated, and it likely plays an important role in the neoplastic phenotype of cancer cells with β-catenin regulatory defects.

Discussion

Mutations in critical components of the Wnt pathway, including β-catenin, APC, and the Axins, play a prominent role in the pathogenesis of human cancer (reviewed in Polakis, 2000; Bienz and Clevers, 2000). Arguably, perhaps the key consequence of the loss-of-function mutations in APC and the Axins or the gain-of-function mutations in β-catenin is to elevate β-catenin levels in the cytoplasm and nucleus. As a result of its dysregulation, β-catenin shows increased ability to bind to TCFs, and altered transcription of TCF-regulated genes ensues. Thus far, it appears that activation of β-catenin/TCF-regulated target genes predominates following Wnt pathway deregulation in cancer, and a number of β-catenin/TCF target genes in cancer have been proposed.

We have presented data here implicating *ITF-2*, particularly transcripts encoding the ITF-2B isoform, as a downstream target of the Wnt/β-catenin/TCF pathway. We found that the rat *ITF-2* gene was consistently activated in β- and γ-catenin transformed RK3E cells, and that 4-OHT-induced activation of a β-catenin-ER fusion protein stimulated *ITF-2* expression, even when new protein synthesis was blocked, suggesting that *ITF-2* is a direct target of β-catenin in the nucleus. Based on studies with a dominant negative form of TCF-4 and reporter gene constructs containing sequences from the region where *ITF-2B* transcripts initiate, evidence was obtained that TCFs play a prominent role in β-catenin's ability to induce *ITF-2* transcription. Moreover, primary ovarian carcinomas with defective β-catenin regulation were found to have elevated *ITF-2B* gene and protein expression when compared to an essentially analogous group of ovarian carcinomas with intact regulation of β-catenin.

Thus far, a quite sizeable number of β-catenin/TCF-regulated genes in cancer have already been proposed. Nevertheless, our data implicating ITF-2 as a β-catenin/TCF target gene are of particular interest. First, the vast majority of the candidate β-catenin/TCF-regulated genes have not been carefully assessed in a large collection of primary human cancers that are well characterized for mutational defects in β-catenin regulation. As such, it is notable that we found that elevated *ITF2* expression was intimately linked to mutational defects in β-catenin regulation in primary ovarian carcinomas. Second, only a minority of the β-catenin/TCF target genes described to date have been implicated as important factors in neoplastic transformation. Two of the candidate TCF targets—*c-MYC* and *cyclin D1*—have been well established to function as oncogenes when deregulated in various settings (reviewed in Sherr, 1996; Dang, 1999),

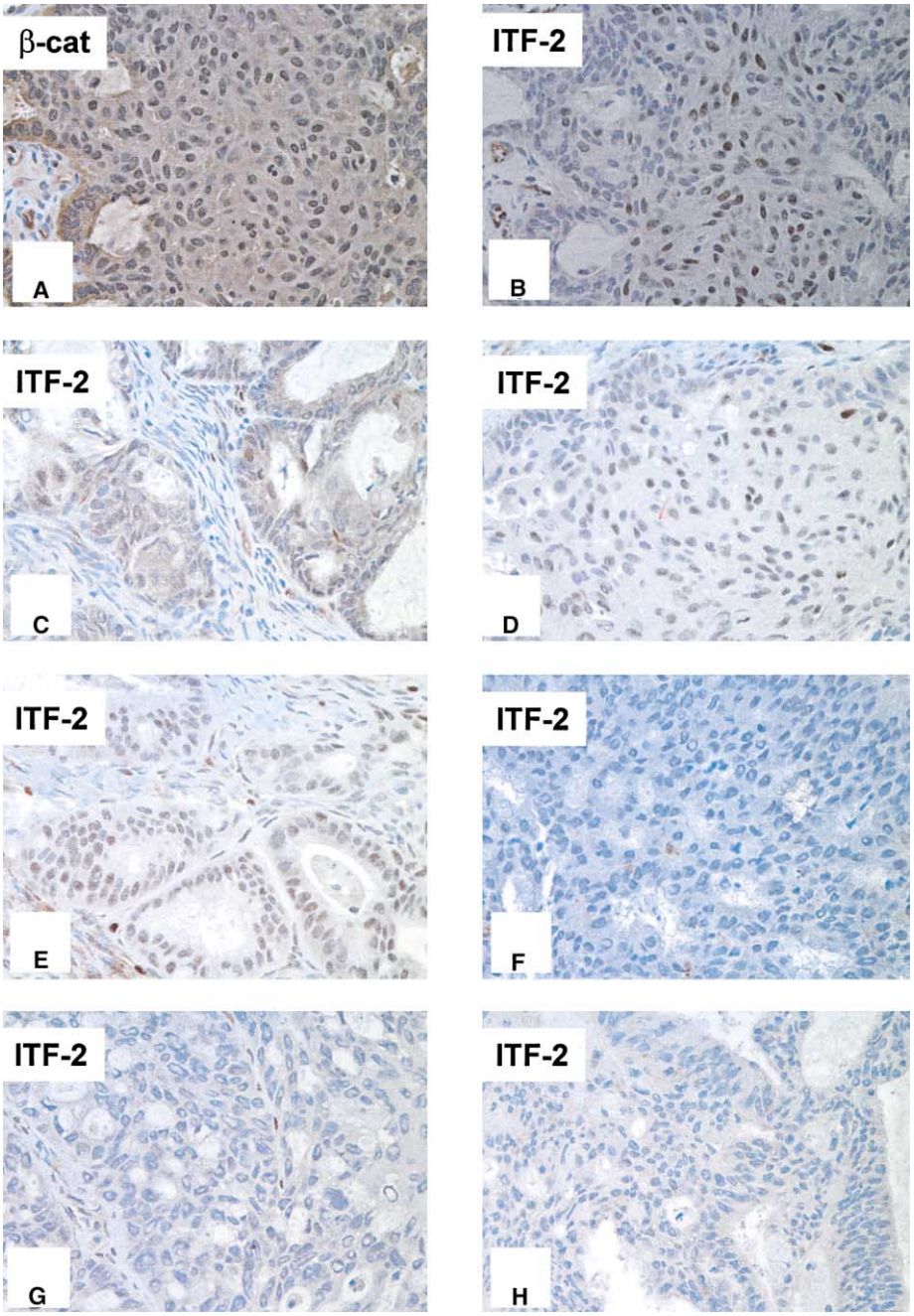

Figure 6. Immunohistochemical staining reveals increased nuclear ITF-2 expression in OEAs with β-catenin regulatory defects compared to OEAs with intact β-catenin regulation

Paraffin-embedded sections were obtained from OEA tumor specimens that had been previously analyzed for *ITF-2A* and *ITF-2B* gene expression, using real-time RT-PCR (Figure 5). The OEA specimens had also been previously studied for β-catenin immunohistochemistry and mutations in critical Wnt pathway components. Photomicrographs are shown of the β-catenin (**A**) and ITF-2 (**B**) staining seen in serial sections of an OEA specimen carrying an oncogenic β-catenin mutation. Representative photomicrographs of ITF-2 staining in other OEAs with defective β-catenin regulation (**C**–**E**) and intact β-catenin regulation (**F**–**H**) are also shown. Note that the nuclear staining for ITF-2 seen in the neoplastic cells of OEAs with β-catenin regulation defects was lacking in OEAs with intact β-catenin regulation, though faint cytoplasmic staining was seen in neoplastic cells of some specimens (e.g., **G** and **H**).

and it is presumed, but not yet proven, that they play significant roles when activated in cancers with β-catenin regulation defects. Two other TCF target genes, namely *MMP-7* and *gastrin*, though not known to be oncogenes based on in vitro or transgenic animal studies, have been implicated via genetic approaches as significant factors in adenoma formation in mice carrying germline *Apc* defects (Wilson et al., 1997; Koh et al., 2000). In light of the rather limited data on the role of candidate TCF target genes in the cancer process, our findings showing that ITF-2B, like mutated β-catenin, has the ability to induce morphological transformation and anchorage-independent growth in RK3E cells is noteworthy. As would be predicted for a downstream target of β-catenin/TCF, *ITF-2B* was readily capable of inducing transformation of RK3E cells expressing a dominant negative mutant of TCF-4. Other candidate β-catenin/TCF-regulated genes that we have studied, including *c-MYC* and *WISP-1*, have not shown focus-forming activity in RK3E cells (F.T.K. and E.R.F., unpublished data), though *c-MYC*'s function as an oncogene in vivo is not in question (Dang, 1999) and *WISP-1* will mediate transformation in certain settings (Xu et al., 2000).

ITF-2 is one of four members of the mammalian class I bHLH proteins (Murre et al., 1994; Massari and Murre, 2000). The other three are HEB and the differentially spliced products of the *E2A* gene, E12 and E47. The class I bHLH proteins display a broad expression pattern, and they are presumed to bind DNA as

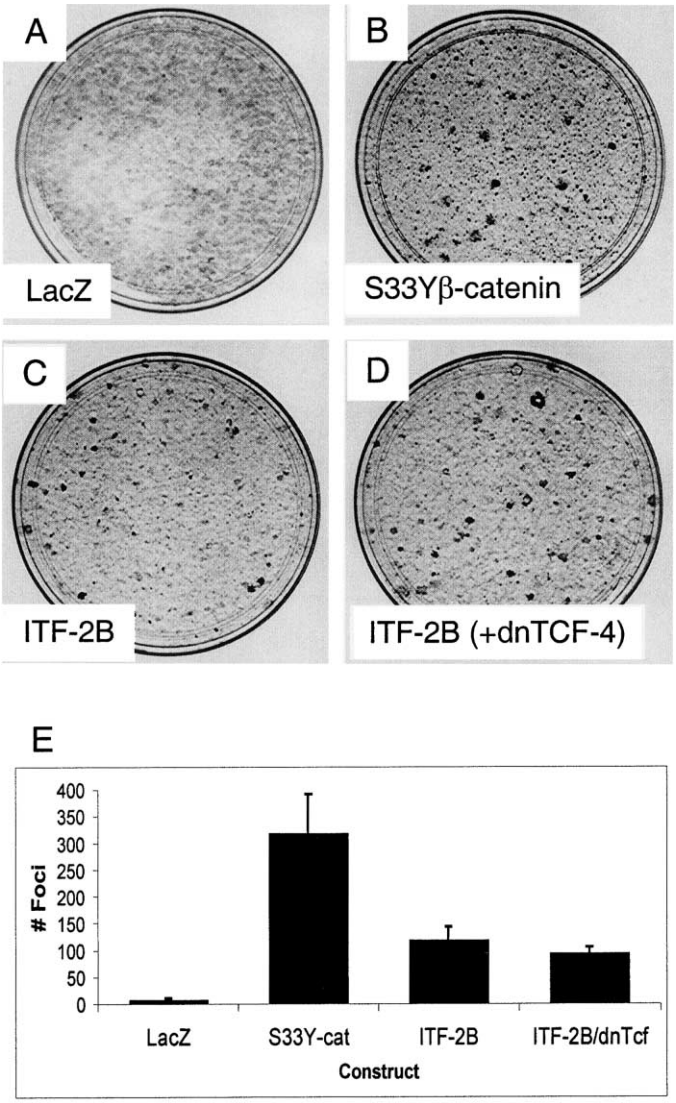

Figure 7. Neoplastic transformation of RK3E cells by ITF-2B

RK3E cells (**A–C**) or RK3E/dnTCF-4 (aka RK3E/Tcf-4ΔN31) cells (**D**) were infected with replication-defective retroviruses containing *LacZ*, *S33Y β-catenin*, or *ITF-2B* cDNAs. Four weeks after infection with the retroviruses, the plates were fixed, stained, and photographed. In **E**, the number of foci (mean and standard deviation) counted in two or more independent experiments with the indicated constructs is shown.

homodimers or following dimerization with one of the many tissue-specific (class II) bHLH proteins, such as the myogenic regulatory factors (e.g., MyoD, MRF-4), heart-specific bHLHs (e.g., dHAND and eHAND), pancreas bHLHs (e.g., BETA2/NEUROD), or neurogenic bHLHs (e.g., MASH-1, Math1, neurogenin) (Massari and Murre, 2000). The Id proteins, because they have the HLH dimerization motif but lack the basic DNA binding region, can inhibit the function of the class I bHLH proteins (Benezra et al., 1990; Massari and Murre, 2000; Norton, 2000). Of the four mammalian Id proteins, two (Id-1 and Id-3) seem to be broadly expressed, whereas the other two (Id-2 and Id-4) show more restricted patterns of expression. It has been suggested that downregulation of Id gene expression accompanied by concordant increases in binding of bHLH proteins to E box sites is critical for differentiation (reviewed in Massari and Murre, 2000; Norton, 2000). Consistent with this model, enhanced expression of Id proteins appears to enhance cell proliferation in several systems. Interestingly, Id-2 is a presumptive downstream target of β-catenin/TCF in colon cancer (Rockman et al., 2001). Id-2's positive role in promoting growth and/or survival in certain settings may be via mechanisms independent of its role in binding to bHLH proteins (Iavarone et al., 1994; Norton, 2000).

Our data implicating *ITF-2B* as β-catenin/TCF-regulated oncogene in epithelial cells are rather unexpected, because prior studies had largely implicated ITF-2 and the other class I bHLH proteins in lineage commitment and differentiation, not oncogenesis (Massari and Murre, 2000). However, altered function of class I and II bHLH proteins has previously been linked to cancer development, since translocations fusing the amino-terminal activation domain of the E2A gene to other transcription factors are seen in B cell leukemias, and translocations activating expression of the class II bHLH proteins SCL/Tal1 and Lyl1 are seen in T cell leukemias (Look, 1997; Massari and Murre, 2000). On the surface, the observation that both Id-2 and ITF-2B appear to be downstream targets of β-catenin/TCF signaling in cancer seems curious. Specifically, because ITF-2 can bind to Id-2, the induction of both proteins by β-catenin/TCF signaling might be predicted to antagonize Id-2's potential role in growth promotion, cell survival, and cancer. Clearly, the interactions among bHLH proteins, Ids, and other non-HLH proteins are complex (Massari and Murre, 2000; Norton, 2000). Additionally, there are many uncertainties about the levels and patterns of expression of class I and class II bHLH proteins and Ids in cancer cells with β-catenin regulatory defects. Given these considerations, it is probably premature to attempt a simple reconciliation of the finding that both *Id-2* and *ITF-2B* are β-catenin/TCF target genes in cancer. Moreover, in light of the important contributions of cell context to phenotypic effects observed, it seems likely that there will be many exceptions to generalizations about the primary role of Id activation in promoting proliferation and the primary role of class I bHLH proteins in lineage commitment and differentiation (Ishiguro et al., 1996; Norton and Atherton, 1998; Chen et al., 1999; Massari and Murre, 2000; Norton, 2000; Parrinello et al., 2001). In fact, because two of the major isoforms of ITF-2—ITF2A and ITF2B—have distinct N termini and apparently distinct functions (Skerjanc et al., 1996; Petropoulos and Skerjanc, 2000), a proposition also supported by their differential regulation by alternative promoters, even generalizations about ITF-2 function, without consideration of the specific isoform of ITF-2 involved, may be unwise. Further studies will undoubtedly enhance understanding of the specific mechanisms by which increased expression of ITF-2B contributes to cancer.

Experimental procedures

Plasmids

Retroviral and/or pcDNA3 expression vectors for mutant β-catenin (codon 33 substitution of tyrosine for serine—S33Y), dominant negative Tcf-4 (Tcf-4ΔN31), and β-galactosidase (*LacZ* gene) have been described (Kolligs et al., 1999). The pBabe-S33Y-ER-puro expression vector encoding a chimeric β-catein/ER protein, in which full-length S33Y β-catenin sequences are fused in-frame to a mutated ER ligand binding domain, was generated by cloning the S33Y β-catenin cDNA into the retroviral plasmid pBABE-puro (Littlewood et al., 1995). pBABE-puro was generously provided by Dr. A. Friedman

(Johns Hopkins University). The reporter constructs pTOPFLASH, which contains three copies of an optimal TCF binding motif (CCTTTGATC), and pFOPFLASH, which contains three copies of a mutant motif (CCTTTGGCC), have been described (Korinek et al., 1997). Plasmid pCH110 (Pharmacia Biosciences, Piscataway, NJ) contains a functional *LacZ* gene cloned downstream of a cytomegalovirus early-region promoter-enhancer element. Rat *ITF-2B* promoter fragments were isolated from a rat genomic library by standard methods, using as a hybridization probe a 250 bp fragment of rat *ITF-2B* cDNA, beginning 550 bp upstream of the start codon. Specific promoter fragments were then generated by PCR from a 4.5 kb subclone from the genomic library screen and ligated into pGL3basic vector (Promega, Madison, WI) for luciferase reporter gene assays. The rat *ITF-2B* cDNA sequence and 5′ genomic flanking sequences were used in BLAST searches to identify the presumptive promoter region for human *ITF-2B*. Specific human ITF-2 genomic fragments were generated by PCR using sequences from GenBank, and the fragments were ligated into pGL3basic vector. Mutations in presumptive TCF DNA binding sites in the proximal region of the rat and human *ITF-2B* promoters were generated by a standard PCR-based mutagenesis approach. A full length human *ITF-2B* cDNA was amplified by PCR from a human colon cancer cDNA and cloned into the retroviral vector pPGS-CMV-CITE-Neo (Kolligs et al., 1999). All plasmid sequences were confirmed by automated sequencing of double-stranded DNA templates.

Cell culture

The HEK293, HT-29, DLD-1, HCT-116, and SW480 cell lines were obtained from American Type Culture Collection (Rockville, MD). The amphotropic Phoenix packaging cell line was obtained from G. Nolan (Stanford University School of Medicine), and the HT-29/β-gal and HT29/APC lines which were obtained from B. Vogelstein (Johns Hopkins University School of Medicine). RK3E, RK3E/Neo, RK3E/Tcf-4ΔN31, and RK3E cells neoplastically transformed by K-ras, β-catenin, and γ-catenin have been previously described (Kolligs et al., 1999, 2000). All cells were grown in 5% $C0_2$ with medium containing 10% fetal bovine serum and penicillin/streptomycin, unless otherwise stated. The 293, Phoenix, and human colon cancer lines, except for HT-29 lines, were grown in DMEM (Life Technologies, Gaithersburg, MD). HT29, HT29/β-Gal, and HT29/APC cells were cultured in McCoy's medium (Life Technologies), and hygromycin B (Sigma, St. Louis, MO) was included at a concentration of 0.6 mg/ml for the HT29/β-Gal and HT29/APC cells. A clonal RK3E cell line expressing the β-catenin S33Y/ER fusion protein was obtained following retroviral transduction of RK3E cells with supernatants from amphotropic Phoenix cells transfected with pBabe-S33Y-ER-puro. Drug selection of pBabe-S33Y-ER-puro-transduced RK3E cells was carried out in puromycin (Sigma, St. Louis, MO) at a concentration of 1.0 μg/ml. A single resistant colony was isolated by ring cloning and expanded into a stable cell line, termed RK3E/S33Y-ER. The RK3E/S33Y-ER line was maintained in 0.5 μg/ml puromycin. To activate the S33Y-ER fusion protein, the RK3E/S33Y-ER cells were treated with media supplemented with 0.5 μM 4-OH-tamoxifen (4-OHT) (Sigma), made from a stock concentration of 100 μM 4-OHT in 100% ethanol. To inhibit new protein synthesis in RK3E/S33Y-ER cells, media was supplemented with cycloheximide (Sigma) at a concentration of 1 μg/ml. To assess effects of wild-type *APC* gene function on *ITF-2A* and *ITF-2B* gene expression, HT29/β-Gal and HT29/APC cells were treated with 150 μM of $ZnCl_2$ for induction of the control *lacZ* and wild-type *APC* genes, respectively. To assess effects of dominant negative TCF-4 on *ITF-2A* and *ITF-2B* gene expression, replication deficient retroviruses encoding TCF-4ΔN31 were used to transduce HT-29 and DLD-1 cells. Empty vector (pPGS-CMV-CITE-Neo) control transductions of the colon lines were carried out in parallel. The TCF-4ΔN31- and empty vector-transduced cells were subsequently selected for 7–10 days in 1.0–1.5 mg/ml G418 (Sigma). Focus formation assays in RK3E cells were carried out for four weeks, as previously described (Kolligs et al., 1999).

Northern blots

Total RNA was extracted using TRIZOL (Gibco BRL). Ten μg of total RNA was separated on 1.2% formaldehyde-agarose gels and transferred to Zeta-Probe GT-membranes (Bio-Rad, Hercules, CA) by capillary action. A 400 bp fragment of the rat *ITF-2* gene, corresponding to sequences present in both the rat *ITF-2A* and *ITF-2B* transcripts, was generated by PCR with primers derived from GenBank accession number U09228, using cDNA prepared from β-catenin-transformed RK3E cells. A 425 bp fragment of the *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was also generated by PCR-based approaches, and both the rat *ITF-2* and *GADPH* fragments were labeled with ^{32}P by random priming. Northern blot hybridization to ^{32}P-labeled probes was carried out by standard methods. Signals were detected by exposure to BioMax-MS film (Kodak, Rochester, NY) at −80°C with an intensifying screen. Quantitation of signals was performed by densitometry.

Real-time RT-PCR assays

Total RNA from cell lines and primary ovarian endometroid adenocarcinomas (OEAs) was treated with RNase-free DNase (Life Technologies, Inc.), and first strand cDNA was synthesized using random hexamers (Pharmacia Biosciences) and Superscript II reverse transcriptase (Life Technologies, Inc.). For PCR with the Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA), 5 ng of cDNA from each sample was used in each reaction. The forward primer for *ITF-2A* was: 5′-ACTATAATGGGAAAGCGG-3′, and the forward primer for *ITF-2B* was: 5′-TCCAGGTTTGCCATCTTCAGT-3′. For both transcripts, the reverse primer was 5′-GCCTGGCGAGTCCCTATTG-3′, and the Taqman probe was 5′-TATGCTCCATCAGCAAGCACTGCCG-3′ (Applied Biosystems), with a carboxyfluorescein label at the Taqman probe 5′ end and a carboxytetramethyl rhodamine label at its 3′ end. Expression of the *hypoxanthine phosphoribosyl transferase* (*HPRT*) housekeeping gene was used to normalize the *ITF-2A* and *ITF-2B* data. For *HPRT*, the forward primer was 5′-TTGCTCGAGATGTGATGAAGGA-3′, and the reverse primer was 5′-CCAGCAGGTCAGCAAAGAATT-3′. The HPRT Taqman probe was: 5′-CCATCACATTGTAGCCCTCTGTGTGCTC-3′ (Applied Biosystems), with 5′ Vic™ and 3′ carboxytetramethyl rhodamine labels. Each sample was run in duplicate and a minimum of two independent experiments were performed for each sample. The following PCR conditions were used: one step of 50°C for 2 min, one step of 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Detection of fluorescence was performed during the run by the ABI system and plotted post-run. The PCRs for *ITF-2A*, *ITF-2B*, and *HPRT* were performed in adjacent wells. Using the software accompanying the Prism 7700 detector, the *HPRT* signals were used for normalization. The Student's t test was used to determine the significance of differences in relative *ITF-2A* and *ITF-2B* expression between the 18 OEAs with strong nuclear staining for β-catenin and mutations in the β-*catenin*, *APC*, *AXIN1*, or *AXIN2* genes and the 27 OEAs lacking strong nuclear β-catenin staining and pathway mutations.

Immunohistochemical analysis

Immunohistochemical analysis of β-catenin and ITF-2 expression in OEAs was performed essentially as described previously (Wu et al., 2001). In brief, 5 μm sections of formalin-fixed, paraffin-embedded tissues were mounted on Probe-On slides (Fisher Scientific, Hanover Park, IL), deparaffinized in xylene, and then rehydrated into distilled water through graded alcohols. Antigen retrieval was enhanced by microwaving the slides in citrate buffer (pH 6.0; Biogenex, San Ramon, CA) for 15 min. Endogenous peroxidase activity was quenched with 6% hydrogen peroxide in methanol, and the slides were blocked with 1.5% normal horse serum for 1 hr. For the β-catenin immunohistochemistry, sections were incubated with a mouse monoclonal anti-β-catenin antibody (C19220, Transduction Laboratories, Lexington, KY) at a dilution of 1:500 overnight at 4°C. Slides were washed in PBS, then incubated with a biotinylated horse anti-mouse secondary antibody for 30 min at room temperature. For the ITF-2 immunohistochemistry, sections were incubated overnight at 4°C with a 1:500 dilution of polyclonal, affinity-purified antibodies raised against a bacterially-expressed, gluthathione-S-transferase fusion protein containing ITF-2B amino acids 461–560 (Ab142), followed by a biotinlylated goat anti-rabbit secondary antibody at a 1:100 dilution for 30 min at room temperature. Antigen-antibody complexes were detected with the avidin-biotin peroxidase method using 3,3′-diaminobenzidine as a chromogenic substrate (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Immunostained sections were lightly counterstained with hematoxylin and then examined by light microscopy.

Luciferase reporter gene assays

Cells were plated in 35 mm dishes 12 hr prior to transfection. Transfections were performed with FuGENE6 (Boehringer Manheim, Indianapolis, IN) according to the manufacturer's protocol, using 2 μl of FuGENE6 per 1 μg of transfected DNA. Analysis of TOPFLASH and FOPFLASH activity in the

RK3E/S33Y-ER cell line was performed as previously described for parental RK3E and β-catenin transformed RK3E lines (Kolligs et al., 1999), except that 24 hr after transfection with reporter constructs, fresh medium containing 0.5 μM 4-OHT was added. Then, at specific time points (e.g., 6 hr, 12 hr, 24 hr), dishes were washed with phosphate buffered saline (PBS), and the cells were scraped from the plates and resuspended in reporter lysis buffer (Promega, Madison, WI). To assess the response of the rat ITF2 promoter constructs to β-catenin, 293 cells were transfected with 0.5 μg pcDNA3/S33Y β-catenin, 0.5 μg pCH110, and 0.5 μg of the ITF2 promoter construct or pGL3basic vector. 48 hr after transfection, the dishes were washed with PBS, and the cells were scraped from the plates and resuspended in reporter lysis buffer. To assess the activity of the human ITF-2 reporter gene constructs in the human colon cancer cell lines and the role of TCFs in their activity, SW480 and HCT116 were cotransfected with 0.5 μg of the human ITF-2 reporter or pGL3basic vectors; 0.5 μg dominant negative TCF (TCF-4ΔN31) in pPGS-CMV-CITE-Neo or 0.5 μg empty pPGS-CMV-CITE-Neo vector; and 0.5 μg pCH110. 48 hr after transfection, the dishes were washed with PBS and the cells were scraped from the plates and resuspended in reporter lysis buffer. Luciferase activities were measured in a luminometer after adding luciferase assay reagent (Promega) to the cell lysates. β-galactosidase activities were determined according to standard methods and were used to control for transfection efficiency.

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