# An autocrine mechanism for constitutive Wnt pathway activation in human cancer cells

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**Summary**

**Autocrine Wnt signaling in the mouse mammary tumor virus model was the first identified mechanism of canonical pathway activation in cancer. In search of this transformation mechanism in human cancer cells, we identified breast and ovarian tumor lines with upregulation of the uncomplexed transcriptionally active form of β-catenin without mutations afflicting downstream components. Extracellular Wnt antagonists FRP1 and DKK1 caused a dramatic downregulation of β-catenin levels in these tumor cells associated with alteration of biological properties and increased expression of epithelial differentiation markers. Colorectal carcinoma cells with knockout of the mutant β-*catenin* allele retained upregulated β-catenin levels, which also could be inhibited by these Wnt antagonists. Together, these findings establish the involvement of autocrine Wnt signaling in human cancer cells.**

## Introduction

Wnt signaling plays a critical role in cell fate determination and tissue development (Nusse and Varmus, 1992; Cadigan and Nusse, 1997). Certain members of this family of secreted glycoproteins interact with coreceptors, Frizzled and LRP5/6, leading to inhibition of β-catenin phosphorylation by the serine threonine kinase, glycogen synthase kinase-β (GSK-3β), within a large cytoplasmic complex including Dishevelled (Dsh), APC, and Axin (Giles et al., 2003). Inhibition of β-catenin phosphorylation impairs its degradation by the ubiquitin/proteosome pathway, resulting in accumulation of the uncomplexed cytosolic molecule. Uncomplexed β-catenin then translocates to the nucleus, where it interacts with TCF/LEF and activates target genes (Giles et al., 2003). Accumulating evidence indicates that signaling through the Wnt canonical pathway regulates the differentiation of adult stem cells in the epithelium of colon (van de Wetering et al., 2002a) and skin (Alonso and Fuchs, 2003), as well as in muscle (Polesskaya et al., 2003) and hematopoietic cells (Reya et al., 2003). Constitutively activated Wnt signaling has also been shown to be causally involved in cancer (Polakis, 2000). *Wnts* were initially identified as a consequence of their transcriptional activation by mouse mammary tumor virus promoter insertion, which initiates mammary tumor formation (Nusse and Varmus, 1992). Later studies established that genetic alterations afflicting *APC* and β-*catenin*, leading to increased uncomplexed β-catenin levels, occur very commonly in human colon and other cancers (Polakis, 2000; Giles et al., 2003). Despite the initial discovery of a Wnt autocrine transforming mechanism in the mouse model more than two decades ago, evidence for this mechanism in human cancer is lacking.

Extracellular inhibitors that function to fine-tune the spatial and temporal patterns of Wnt activity and act at the cell surface to inhibit Wnt signaling through its receptors have recently been discovered (Kawano and Kypta, 2003). One group of Wnt antagonists is the secreted Frizzled-related proteins (FRPs), which share sequence similarity with the Frizzled receptor CRD (cysteine rich domain), but lack the transmembrane and intracellular domains (Leyns et al., 1997; Wang et al., 1997; Finch et al., 1997). Through its CRD, FRP exhibits the ability to bind Wnt, form dimers, and heterodimerize with Frizzled (Leyns et al., 1997; Wang et al., 1997; Rattner et al., 1997; Lin et al., 1997; Bafico et al., 1999). Thus, FRP may act not only to sequester Wnts but also to inhibit Wnt signaling via formation of nonfunctional complexes with the Frizzled receptor. Another potent Wnt antagonist, designated Dickkopf-1 (DKK1), is the prototype of a family of secreted proteins structurally unrelated to Wnt or Frizzled (Glinka et al., 1998; Fedi et al., 1999). DKK1 binds the Wnt coreceptor LRP6 and causes its endocytosis through formation of a ternary complex with the transmembrane protein Kremen (Mao et al., 2001, 2002; Bafico et al., 2001; Semenov et al., 2001). Since LRP6 has been shown to activate only the Wnt/β-catenin pathway (Pinson et al., 2000; Wehrli et al., 2000), the interaction of DKK1 with LRP6 establishes DKK1 as a specific antagonist of canonical Wnt signaling. The availability of specific antagonists that inhibit Wnt signaling at the level of

SIGNIFICANCE

**The highly conserved Wnt canonical signaling pathway plays a crucial role in normal development and in human tumorigenesis. By use of specific antagonists that inhibit Wnt ligand signaling through its receptors, we identified a functional Wnt autocrine loop in human breast and ovarian carcinoma cells. An increasing number of cancer therapeutic agents are being directed against ligands or their cell surface receptors. Thus, autocrine Wnt signaling could provide a novel target for therapeutic intervention by means of Wnt antagonists or other modalities that interfere with cell surface interactions of Wnts and their receptors.**

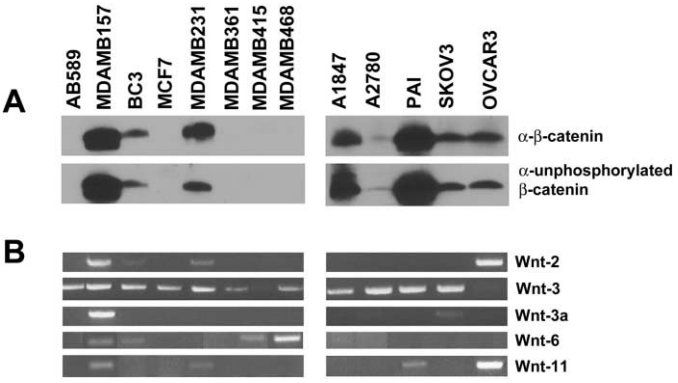


**Figure 1.** Wnt signaling upregulation in human tumor breast and ovarian cancer cell lines

**A:** Analysis of uncomplexed and unphosphorylated β-catenin in human tumor cells. Around 1 mg of total cell lysates was subjected to the GST-E-cadherin assay (Bafico et al., 1998). Following SDS-PAGE analysis, uncomplexed β-catenin was detected by an anti-β-catenin antibody (upper panel). An antiserum specific to dephosphorylated β-catenin was utilized to detect the uncomplexed unphosphorylated form of the protein (lower panel).

**B:** RT-PCR analysis of Wnt ligands. RNA from each tumor cell line was reverse transcribed and amplified with primers specific for each indicated Wnt gene (see Experimental Procedures). Amplification was performed for 35 cycles using the following profile: 94°C for 30 s, optimized annealing temperature for 30 s, and 72°C for 30 s. PCR products were resolved on a 2% agarose gel and visualized by ethidium bromide staining.

**Table 1.** Wnt signaling upregulation in human tumor cell lines.

| Cell line | Uncomplexed β-catenin level | Inhibition by FRP and/or DKK |
|---|---|---|
| Breast | | |
| AB589 | − | ND |
| BC3 | + | + |
| MDAMB134 | − | ND |
| MDAMB157 | ++++ | + |
| MDAMB175 | − | ND |
| MDAMB231 | ++ | + |
| MDAMB361 | − | ND |
| MDAMB415 | − | ND |
| MDAMB435 | − | ND |
| MDAMB453 | − | ND |
| MDAMB468 | − | ND |
| MCF7 | − | ND |
| Ovarian | | |
| OVCAR3 | + | − |
| A1847 | ++ | + |
| A2780 | − | ND |
| SKOV3 | + | +/− |
| 44S | + | +/− |
| PAI | ++++ | + |
| 26S | − | ND |
| 53S | +++ | − |

Human tumor cell lines without detectable APC or β-catenin mutations were analyzed for expression of uncomplexed β-catenin as described in the Experimental Procedures. Relative levels were approximated based on comparison between different lines analyzed at the same time (see Figure 1). Inhibition by FRP1 and/or DKK1 was scored as positive based on the results of at least three independent experiments. (ND= not determined).

interaction with its surface receptors provided us with the means to investigate whether Wnt autocrine signaling could be implicated in human tumorigenesis.

## Results

### Identification of human breast and ovarian tumor cell lines with constitutive Wnt pathway activation

To search for evidence of autocrine Wnt signaling in human tumors, we initially surveyed a panel of human breast and ovarian tumor cell lines for increased levels of uncomplexed β-catenin. Several were identified as positive by this approach (Figure 1A and Table 1). Activation of Wnt signaling specifically increases the levels of N-terminally unphosphorylated β-catenin, which represents the transcriptionally active form of the protein (van Noort et al., 2002; Staal et al., 2002). Analysis of the uncomplexed β-catenin pool with an antibody that specifically recognizes the unphosphorylated form revealed that this pool in each case contained the transcriptionally active form (Figure 1A). No detectable oncogenic lesions in either β-catenin or APC, the most frequently altered cancer genes in this pathway (Polakis, 2000; Bienz and Clevers, 2000), were found in any of these β-catenin upregulated tumor lines (data not shown), suggesting a novel mechanism. In an effort to implicate Wnt autocrine signaling, we analyzed expression of representative *Wnts* by RT-PCR using primers specific for each. Figure 1B shows that the Wnt ligands analyzed exhibited different patterns of expression, and that the tumor cell lines containing upregulated β-catenin expressed one or more of these Wnts.

### Wnt antagonists identify autocrine Wnt signaling in human tumor cells

To directly address the possibility of an autocrine signaling loop in these cells, we took advantage of the FRP1 and DKK1 antagonists, which inhibit Wnt signaling at the level of ligand/receptor interactions (Leyns et al., 1997; Wang et al., 1997; Bafico et al., 1999, 2001; Mao et al., 2001; Semenov et al., 2001). As shown in Figure 2A, addition of DKK1 to NIH3T3 cells stably expressing Wnt-3a resulted in striking inhibition of upregulated β-catenin levels. In contrast, the same inhibitor had no effect on uncomplexed β-catenin induced by exogenous β-catenin expressed under the control of a tet regulatable promoter. Thus, we reasoned that if a Wnt autocrine loop were functional in human tumor cells, FRP1 or DKK1 antagonists should cause specific inhibition of upregulated β-catenin levels.

As shown in Figure 2B, exposure of MDAMB157 breast tumor cells to increasing concentrations (0.5–10 nM) of purified DKK1 (Bafico et al., 2001) led to a dose-dependent, marked reduction in the levels of uncomplexed β-catenin (Figure 2B). Stable expression of FRP1 or DKK1 by retroviral-mediated transduction in MDAMB157 cells led to a dramatic reduction in uncomplexed β-catenin levels as well (Figure 2C). Inhibition of constitutively upregulated β-catenin was also observed with expression of FRP1 or DKK1 in several other breast and ovarian tumor cell lines, including MDAMB231, A1847, and PAI (Figure 2D). The expression of Flag-tagged DKK1 and FRP1 proteins in each of these cell lines was confirmed by immunoblot analysis. Wnt signaling activates TCF dependent transcription, which can be monitored by reporters containing TCF-responsive elements (Morin et al., 1997). DKK1 caused a striking reduction in the level of endogenous TCF-dependent signaling in a representative ovarian tumor cell line, PAI (Figure 2E). These findings further established that TCF-dependent transcription was constitutively activated in these tumor cells by an autocrine Wnt mechanism. As summarized in Table 1, three of eleven breast

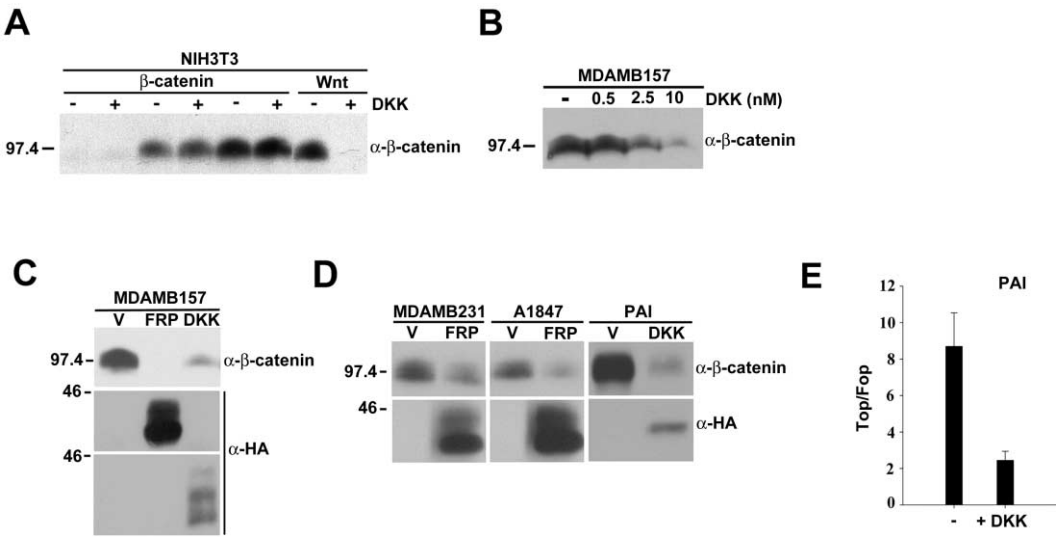


**Figure 2.** FRP1 and DKK1 inhibition of autocrine Wnt signaling in human tumor cell lines

**A:** DKK1 acts at the level of ligand/receptor interactions. NIH3T3 cells expressing β-catenin under the control of the Tet inducible promoter were grown for 4 days in the presence of different amounts of tetracycline (lanes 1 and 2: 1 μg/ml; lanes 3 and 4: 7.5 ng/ml; lanes 5 and 6: 5 ng/ml). These cells as well as Wnt expressing NIH3T3 cells were incubated with purified DKK1 (10 nM) (Bafico et al., 2001) and solubilized after 2 hr. 1 mg of total cell lysate was subjected to the GST-E-cadherin binding assay, followed by SDS-PAGE and immunoblot analysis with the anti-β-catenin antibody.

**B:** Soluble DKK1 inhibits upregulated β-catenin levels in MDAMB157 cells. Cultures were exposed to increasing concentrations of purified DKK1 for 2 hr, solubilized, and analyzed for uncomplexed β-catenin as described above.

**C:** MDAMB157 breast cancer cells were infected with either empty vector or FRP1-HA or DKK1-HA retroviruses and marker selected (top panel). Expression of tagged FRP1 was assessed by immunoblot analysis of lysates with an anti-HA antibody (middle panel). The same antiserum was utilized to detect DKK1 in serum-free concentrated (5×) conditioned media (lower panel).

**D:** Analysis of breast (MDAMB231) and ovarian (A1847, PAI) tumor cell lines for uncomplexed β-catenin (upper panel) or HA-tagged FRP1 and DKK1 (lower panel).

**E:** DKK1 inhibition of TCF-reporter transcriptional activity in PAI tumor cells. Cells were cotransfected with either TOP-Glow or Fop-Glow plasmids, and the pCMV-RL plasmid encoding Renilla luciferase as an internal control for transfection efficiency. The values represent the mean (±SD) of two independent experiments, and the ratio of the activity obtained with the wild-type TOP-Glow plasmid to the activity observed with the mutant FOP-Glow plasmid is shown.

tumor cell lines exhibited upregulated β-catenin, which in each case was inhibited by FRP1 and/or DKK1. Two of eight ovarian tumor cell lines demonstrated uncomplexed β-catenin levels, which were decreased in response to the antagonists. A high level of upregulated β-catenin was detected in 53S tumor cells but showed no detectable response to the inhibitors, implying a lesion in the canonical pathway other than an autocrine loop.

### Inhibition of autocrine Wnt signaling by siRNA directed against *LRP6*

In an effort to independently confirm the existence of an autocrine Wnt signaling loop, we generated siRNAs specific for *LRP5* and *LRP6*, the Wnt coreceptors specific for the canonical pathway (Pinson et al., 2000; Wehrli et al., 2000). As shown in Figure 3A, the exogenous expression of each LRP receptor in 293T cells was specifically inhibited by the homologous but not the heterologous siRNA. When the same siRNAs were expressed in 293T cells treated with Wnt-3a conditioned media, we observed that *LRP6* siRNA caused a reduction in Wnt induced uncomplexed β-catenin levels, while *LRP5* siRNA had no detectable effect (Figure 3B, left panel). These results implied that canonical signaling in response to Wnt-3a in these cells required endogenous LRP6. There was no effect of either siRNA on uncomplexed β-catenin levels in 293T cells expressing mutant β-catenin under the same conditions (data not shown). We next tested the effects of these same siRNAs on PAI tumor cells and observed that *LRP6* but not *LRP5* siRNA caused a marked inhibition in uncomplexed β-catenin levels (Figure 3B, right panel). These results provide strong evidence, independent of the use of Wnt antagonists, that constitutive Wnt signaling was due to an autocrine loop in these human tumor cells, and implicated LRP6 as the specific Wnt canonical receptor involved.

### Effects of Wnt autocrine signaling inhibition on tumor cell phenotype

Exogenous expression of Wnts that signal through the canonical pathway in mammalian cells causes acquisition of properties associated with the transformed phenotype (Blasband et al., 1992; Wong et al., 1994; Shimizu et al., 1997; Bafico et al., 1998; Orford et al., 1999). For example, stable expression of transforming Wnts in responsive cells induces increased saturation density, which can be specifically blocked by stable coexpression of FRP1 or DKK1 (Bafico et al., 1998; Fedi et al., 1999). There is also evidence that Wnt signaling can inhibit apoptosis (Chen et al., 2001; You et al., 2002; Longo et al., 2002). Having identified human tumor cells with autocrine Wnt signaling, we analyzed the effects of FRP1 or DKK1 on these biological properties. As shown in Figure 4A, MDAMB157 cells overexpressing FRP1 or DKK1 exhibited decreased saturation density when compared to vector-transduced parental cells. To confirm that the effects

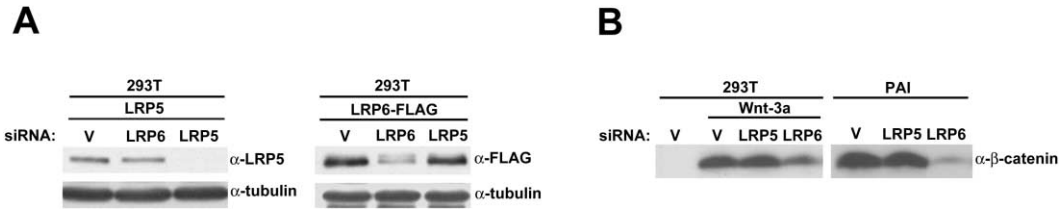


**Figure 3.** Effects of *LRP5* and *LRP6* siRNAs on Wnt signaling

**A:** Depletion of LRP5 and LRP6 by specific siRNAs. 19 nt siRNAs were generated against the extracellular domain of human *LRP5* and *LRP6* as described in the Experimental Procedures. 293T cells were transiently transfected with 1 μg of each siRNA construct along with 0.5 μg of LRP5 or LRP6-FLAG. At 72 hr, LRP5 protein levels were analyzed with an LRP5 polyclonal antibody (Orbigen), while LRP6 levels were detected utilizing an anti-FLAG antibody. The same lysates were analyzed with an anti-tubulin antibody as a loading control.

**B:** Effects of *LRP5* and *LRP6* siRNAs on Wnt-3a-stimulated 293T cells or autocrine Wnt PAI human tumor cells. Left: subconfluent 293T cells were transiently transfected with 2.5 μg of each siRNA construct. After 72 hr, cells were treated for two hours with either control or Wnt-3a conditioned media and analyzed for uncomplexed β-catenin levels. Right: subconfluent PAI tumor cells were transiently transfected with 2.5 μg of each siRNA and analyzed for uncomplexed β-catenin levels at 72 hr.

of FRP1 and DKK1 on MDAMB157 were through inhibition of Wnt function, we infected the immortalized human mammary epithelial cell line, AB589 (Stampfer and Bartley, 1985), which exhibited undetectable levels of uncomplexed β-catenin, (Figure 1A) with either vector or FRP1 or DKK1 retroviruses. Expression of the inhibitors in these cells resulted in no detectable effect on saturation density (data not shown).

To assess the effects of Wnt inhibition by FRP1 or DKK1 on the response of MDAMB157 cells to apoptotic stimuli, we exposed the cells to increasing concentrations of tert-butyl hy-

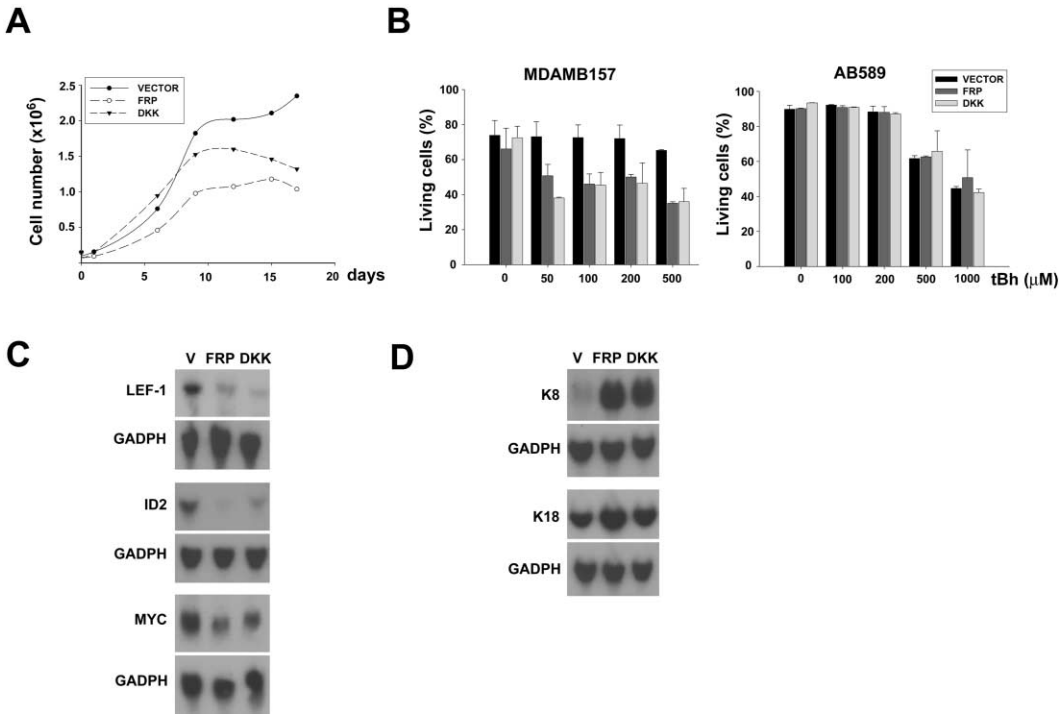


**Figure 4.** Functional effects of FRP1 and DKK1 inhibition of autocrine Wnt signaling in human breast tumor cells

**A:** FRP1 and DKK1 effects on saturation density. MDAMB157 cells exogenously expressing either vector control, FRP1, or DKK1 were transferred at $1.5 \times 10^5$ cells per well in 6-well plates. Cell counts were performed in duplicate at the indicated times, and values represent the mean of two independent experiments.

**B:** Effects of FRP1 and DKK1 on apoptosis in response to an oxidative stress inducer. Subconfluent cultures of MDAMB157 or AB589 were treated with the indicated amounts of tBh and subjected to FACS analysis at 24 hr as described in the Experimental Procedures. The values obtained are expressed as the mean ± SD of two independent experiments performed in duplicate.

**C and D:** FRP1 and DKK1 effects on Wnt target genes and differentiation markers. 40 μg of total RNA extracted from MDAMB157 cells exogenously expressing vector, FRP1, or DKK1 were resolved on a 1% agarose gel and transferred to a nylon membrane. Hybridization was performed with $\alpha$-$^{32}$P-dCTP labeled probes as indicated. For *LEF-1* and *c-myc*, the full-length cDNAs were labeled; for *Id2*, *keratin 8,* and *keratin 18*, probes were generated by RT-PCR. For normalization, membranes were stripped and reprobed with a commercially available GADPH probe.

droperoxide (tBh), a known inducer of oxidative stress (Macip et al., 2003), and analyzed the apoptotic response. At each concentration of tBh analyzed, there was a statistically significant increase in the level of apoptosis in the presence of FRP1 or DKK1 overexpression (Figure 4B, left panel). Similar experiments performed with AB589 cells expressing FRP1 or DKK1 revealed no detectable differences in their apoptotic responses compared to the vector infected cells (Figure 4B, right panel). We noted that levels of expression of these antagonists decreased with passage of the transfected and marker selected tumor cells. This made it difficult to study their effects on tumor formation in vivo and suggested a negative selective pressure in tissue culture against these antagonists in Wnt autocrine tumor cells.

Alterations in the expression of a number of genes identified as targets of canonical Wnt signaling have been reported, although it should be noted that there is variability among Wnt target genes in different cell systems (Giles et al., 2003). To investigate the effects of FRP1 and DKK1 inhibition on gene expression in MDAMB157 breast tumor cells, we performed Northern blot analysis of representative Wnt transcriptional targets, including *myc* (He et al., 1998), the *LEF-1* transcription factor (Filali et al., 2002), and the dominant negative helix-loop-helix transcriptional regulator, *Id2* (Rockman et al., 2001). Of note, the expression of each was reduced by stable expression of FRP1 or DKK1 (Figure 4C). We did not observe significant changes in the levels of another Wnt target gene, *cyclin D1* (Tetsu and McCormick, 1999; Shtutman et al., 1999) under the same conditions (data not shown). However, it should be noted that Wnt can induce mammary tumors in the mouse in a *cyclin D1*-null genetic background, implying that in this tissue at least, *cyclin D1* is unlikely to be a critical Wnt target (Yu et al., 2001; Rowlands et al., 2003).

Since canonical Wnt signaling appears to be involved in maintaining epithelial progenitor cells of several tissues (van de Wetering et al., 2002b; Alonso and Fuchs, 2003; Reya et al., 2003; Polesskaya et al., 2003), including the mouse mammary gland (Li et al., 2003; Liu et al., 2004), we also investigated whether downregulation of Wnt signaling in MDAMB157 tumor cells affected their differentiated state. RNA blot analysis with probes for *keratins 8* and *18*, two markers known to be expressed by differentiated mammary epithelial cells (Stingl et al., 2001; Going, 2003), revealed that stable expression of FRP1 or DKK1 led to a striking increase in the expression of *keratin 8* as well as an increase in *keratin 18* levels (Figure 4D). All of these findings provide evidence for a Wnt autocrine transforming mechanism in human tumor cells.

### Effects of Wnt antagonists on HCT116 colon cancer cells with knockout of either wild-type or mutant β-catenin

Physiological Wnt signaling appears to be required for maintenance of the crypt progenitor phenotype in colonic epithelium (Pinto et al., 2003; Kuhnert et al., 2004). Recent findings that *FRP1* is mutated or methylated in a high fraction of colon carcinomas (Suzuki et al., 2002; Caldwell et al., 2004) led us to investigate whether a contribution of Wnt autocrine signaling in such tumors might be masked by mutations in downstream components of this pathway. For this purpose, we took advantage of the HCT116 colorectal cancer cell line, which harbors a β-*catenin* mutation (Morin et al., 1997). HCT116 clones have been engineered by homologous recombination to contain either the wild-type or mutant β-*catenin* allele (Sekine et al., 2002; Chan et al., 2002). RT-PCR analysis revealed expression of canonical Wnt ligands, including the highly transforming Wnt-3a in HCT116 cells (Figure 5A). Moreover, the wt allele-containing clone retained high levels of uncomplexed β-catenin, indicating constitutive upregulation of the Wnt pathway independent of the presence of the mutant β-*catenin* allele (Figure 5B). Similar results were observed with other wt β-*catenin* allele-containing clones (data not shown). Whereas FRP1 expression had little if any effect on uncomplexed β-catenin levels in the clone containing only the mutant allele, there was a dramatic reduction in β-catenin levels in the wild-type β-*catenin* allele-containing clone (Figure 5B). These findings established that an autocrine Wnt loop must exist in HCT116 cells.

We next assessed the effects of FRP1 inhibition on the expression of *Id2*, *Cyclin D1*, and *Myc*, known targets of Wnt/β-catenin in colorectal cancer (Rockman et al., 2001; Tetsu and McCormick, 1999; He et al., 1998). Northern blot analysis revealed high levels of *Id2* expression in parental HCT116 as well as in the β-*catenin* mutant allele clone. The wild-type β-*catenin* allele-containing clone also showed *Id2* expression at a somewhat lower level, consistent with the relative levels of uncomplexed β-catenin observed in these clones (Figures 5B and 5C). Of note, FRP1 expression led to reduction in the *Id2* transcript level in the wild-type β-*catenin* but not in the mutant allele-containing clone. Similarly, expression of *Cyclin D1* and *Myc* in the wild-type allele-containing clone was dramatically inhibited by FRP1 expression (Figure 5D). These results were consistent with the ability of FRP1 to inhibit TCF-dependent reporter activity in the β-*catenin* wild-type allele clone (Figure 5E). To assess the effects of Wnt autocrine inhibition in vivo, we performed tumorigenicity experiments utilizing parental or β-*catenin* wild-type allele-containing HCT116 cell lines, in which we were able to obtain stable marker selected mass cultures expressing FRP1. Of note, while FRP1 expression had no effect on tumor growth induced by parental cells, it caused a striking reduction in the tumor forming ability of β-*catenin* wild-type allele-containing cells (Figure 5F). All of these findings establish that autocrine Wnt signaling can be present in human colon carcinoma cells that harbor downstream lesions within the canonical pathway.

## Discussion

Our present studies establish constitutive upregulation of β-catenin in human tumor cells by a novel mechanism involving Wnt autocrine signaling. This mechanism was initially discovered in the mouse mammary tumor model in which MMTV promoter insertion oncogenically activates *Wnt* expression (Nusse and Varmus, 1992). Later studies identified mutations in downstream components resulting in upregulation of this pathway in human colon carcinomas and a variety of other tumors (Polakis, 2000; Giles et al., 2003). We observed several human breast and ovarian tumor cell lines, which exhibited Wnt ligand expression and increased levels of the transcriptionally active form of unphosphorylated, uncomplexed β-catenin without detectable lesions in commonly implicated downstream signaling components, *APC* or β-*catenin*. Findings that FRP1 and DKK1, two specific antagonists of Wnt signaling at the level of ligand/receptor interactions (Leyns et al., 1997; Wang et al., 1997; Mao et al., 2001, 2002; Bafico et al., 2001), caused downregulation

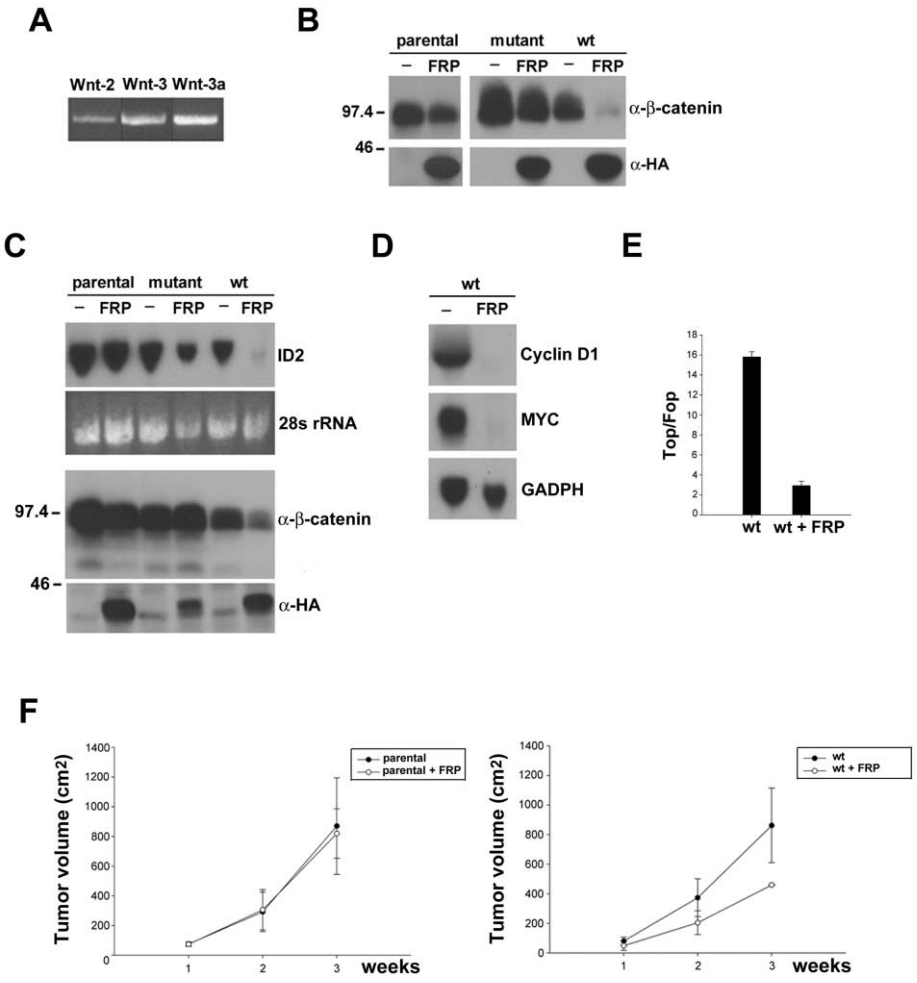


**Figure 5.** Wnt autocrine signaling in HCT116 human colon cancer cells

**A:** RT-PCR analysis of expression of Wnt ligands in HCT116 colorectal cancer cells. RNA was reverse transcribed and amplified with primers specific for each indicated Wnt as described in the Experimental Procedures. PCR product were resolved on 2% agarose gel and visualized by ethidium bromide staining.

**B:** Effects of FRP1 on uncomplexed β-catenin levels in HCT116 parental and allele-targeted clones. HCT116 parental and clones expressing either wt or mutant β-*catenin* allele were transfected with FRP1-HA and subjected to the GST-E-cadherin binding assay. Uncomplexed β-catenin was detected with anti-β-catenin antibody (upper panel). The levels of FRP1 in the lysates were detected with an anti-HA antibody (lower panels).

**C:** Effects of FRP1 on the *Id2* target gene expression. Top: Northern blot analysis of *Id2*. RNA was extracted from HCT116 parental, and either wt or mutant β-*catenin* allele-containing clones transfected with either vector control or FRP1-HA. 40 μg of total RNA was resolved on a 1% agarose gel, transferred to a nylon membrane, and hybridized with a RT-PCR-generated *Id2* probe labeled with $^{32}$Pα-dCTP (see Experimental Proceduress and legend to Figure 4C). 28S ribosomal RNA was utilized as loading control. Bottom: Analysis of uncomplexed β-catenin and FRP1 levels. As controls for FRP1 function, cell lysates were obtained at the same time as the RNA extraction and analyzed as in **B**.

**D:** FRP1 inhibits *Cyclin D1* and *Myc* gene expression in the wild-type allele-containing HCT116 clone. Hybridization was performed utilizing $^{32}$Pα-dCTP-labeled cDNAs for either *Cyclin D1* or *c-myc*. For normalization, membranes were stripped and hybridized with a GADPH probe.

**E:** Effects of FRP1 on TCF-reporter transcriptional activity in the wild-type allele-containing HCT116 clone. Cells were transfected and analyzed as described in the Experimental Procedures and in the legend to Figure 2E. The ratio of the reporter activity obtained with the wild-type TOP-Glow plasmid to the activity observed with the mutant FOP-Glow plasmid is shown.

**F:** FRP effects on tumor formation. The indicated cell lines were subcutaneously injected into nude mice at 2.5 × $10^6$ per site. Tumor size was monitored weekly, and values represent the mean (±SD) of 4 inoculation sites per cell line.

of uncomplexed β-catenin levels in these tumor cells strongly implicated an autocrine Wnt loop. Independent evidence in support of this conclusion derived from the use of siRNAs directed against *LRP5* and *LRP6*. These studies established in PAI ovarian cancer cells that LRP6 was specifically responsible for transducing the Wnt autocrine signal. Moreover, functional studies with a human breast tumor cell line revealed that Wnt antagonists inhibited known Wnt-induced biological effects as well as Wnt target gene expression. All of these findings imply that autocrine Wnt signaling plays a role in the etiology of some human tumors.

Previous studies have detected *Wnt* transcript expression in human breast tumors (Huguet et al., 1994; Dale et al., 1996; Bui et al., 1997). There has also been at least one report of cytoplasmic/nuclear staining of β-catenin in breast cancer tissues (Lin et al., 2000), whereas mutations in downstream components of the canonical pathway have not been reported (Brown, 2001; Giles et al., 2003). β-*catenin* mutations have been observed in ovarian carcinomas of the endometroid type, which reflects around 10%–20% of ovarian malignancies (Palacios and Gamallo, 1998; Sagae et al., 1999; Wu et al., 2001). Our present studies identified autocrine Wnt signaling, as defined by the ability of the Wnt antagonists FRP1 and/or DKK1 to cause downregulation of activated β-catenin, in around 25% of human breast and ovarian cancer cell lines analyzed, implicating this mechanism in a significant fraction of such tumors. Whether autocrine Wnt signaling in these tumor cells results from Wnt misexpression, aberrant upregulation of some other components such as a specific coreceptor not normally present, or downregulation of Wnt antagonists remains to be elucidated.

Recent evidence that *FRP* genes are frequently hypermethylated in colon carcinomas (Suzuki et al., 2002; Caldwell et al., 2004) suggested the possibility that autocrine Wnt signaling might also play a role in the development of tumors which exhibit constitutive pathway activation due to mutations in downstream components. We showed that HCT116 tumor cells, harboring both a β-*catenin* mutation (Morin et al., 1997) and FRP hypermethylation (Suzuki et al., 2002; Chan et al., 2002), expressed Wnt ligands. Moreover, upregulated β-catenin levels observed in HCT116 clones with targeted inactivation of the mutant allele

(Sekine et al., 2002) were specifically downregulated by exogenous FRP. Finally, inhibition of Wnt autocrine signaling in wt allele-containing HCT116 cells led to decreased expression of Wnt transcriptional targets expressed in colon carcinoma cells with constitutive activation of the canonical pathway (He et al., 1998; Rockman et al., 2001; Tetsu and McCormick, 1999). These findings establish the presence of a Wnt autocrine loop in HCT116 cells and may help to explain why small molecule antagonists of the oncogenic TCF/β-catenin complex were similarly potent in growth inhibition of wt and mutant allele-containing HCT116 clones (Lepourcelet et al., 2004).

Accumulating evidence indicates that Wnt signaling is involved in the self-renewal of intestinal epithelial stem/progenitors as well as several other progenitor cell types (van de Wetering et al., 2002b; Reya et al., 2003; Alonso and Fuchs, 2003). For example, DKK1 overexpression caused impaired differentiation and architectural degeneration of mouse intestinal epithelium (Pinto et al., 2003; Kuhnert et al., 2004). Conversely, upregulated Wnt signaling can confer an immortalized stem/progenitor phenotype, as demonstrated by findings that expression of a dominant negative TCF4 in certain colorectal cancer lines induced a colon epithelial differentiation program (van de Wetering et al., 2002b). While this manuscript was in preparation, Suzuki et al. (2004) reported the presence of FRP hypermethylation in colorectal adenomas as well as in earlier lesions, monoclonal aberrant crypt foci (ACF), which usually lack *APC* mutations (Siu et al., 1999). Taken together with these results, our findings are consistent with the concept that Wnt autocrine activation may be an early event that expands a colon crypt progenitor population and sets the stage for subsequent β-*catenin* or *APC* lesions. Whereas we observed that FRP like DKK had little or no effect on Wnt signaling activation induced by either overexpressed or mutated β-catenin, these authors reported that exogenous FRP attenuated Wnt signaling in colon tumor cells with either β-*catenin* or *APC*. The basis for these differences remains to be resolved.

An autocrine transformation mechanism was initially identified through the discovery that the *sis* retroviral oncogene encoded platelet-derived growth factor (PDGF) (Doolittle et al., 1983; Waterfield et al., 1983), and subsequent findings have implicated this mechanism in naturally occurring human cancers (Bafico and Aaronson, 2003; Blume-Jensen and Hunter, 2001). It is known that activation of receptor tyrosine kinases by growth factors can occur within the secretory pathway. Whether signaling is functional by a so-called intracrine mechanism within the secretory pathway is not resolved (Huang et al., 1984; Keating and Williams, 1988; Bejcek et al., 1989; Lee and Donoghue, 1992; Wiley et al., 1998). In Wnt autocrine tumor cells, we demonstrated that soluble DKK1 caused a striking reduction in upregulated uncomplexed β-catenin to essentially undetectable levels, analogous to effects observed with Wnt transformed mouse cells. Since soluble DKK1 acts only at the cell surface, these findings exclude an intracrine component for Wnt autocrine signaling. An increasing number of cancer agents have successfully targeted ligands or receptors at the cell surface (Hudziak et al., 1989; Myers et al., 1992; Slamon et al., 2001). Thus, autocrine Wnt signaling could provide a novel target for therapeutic intervention with DKK1 and FRP1 antagonists or other modalities, aimed at interfering with cell surface interactions involving Wnts and their receptors.

## Experimental procedures

### Constructs

Human *FRP1* (Bafico et al., 1999) and *DKK1* (Bafico et al., 2001) cDNAs were subcloned into a pBabe-derived retrovirus vector containing a carboxy-terminal HA tag and were cotransfected with the pCL-ampho packaging plasmid into 293T cells. Culture fluids were harvested at 72 hr and titrated on NIH3T3 cells. The β-*catenin* cDNA, generously provided by Dr. W. Birchmeier (Hulsken et al., 1994), was expressed under the control of a Tet regulatable promoter using a system we have previously reported (Sugrue et al., 1997). pCMV-LRP6-Flag has been previously described (Liu et al., 2003). Human *LRP5*, generously provided by Dr. Matthew Warman (Case Western Reserve University), was subcloned into pcDNA3.1 (Invitrogen).

### Cell culture and gene transduction

Human tumor cell lines including breast (MDAMB157, BC3, MCF7, MDAMB231, MDAMB134, MDAMB175, MDAMB435, MDAMB453, MDAMB361, MDAMB415, and MDAMB468), ovarian (A1847, A2780, PAI, SKOV3, OVCAR3, 44S, 53S, 26S, and OV90) and colon HCT116 were maintained in Dulbecco's modified Eagle's (DMEM) medium supplemented with 10% fetal bovine serum. The immortalized mammary epithelial cell line AB589 (Stampfer and Bartley, 1985) was cultured in the same media with the addition of 1 μM dexamethasone. HCT116 allele-targeted clones engineered by homologous recombination have been described (Sekine et al., 2002) and were maintained in DMEM containing 2 μg/ml puromycin. NIH3T3 cells were maintained in DMEM medium supplemented with 10% calf serum. For retroviral mediated gene transduction, cultures were plated at $5 \times 10^5$ cells per 60 mm plate in growth media containing 2 μg/ml of polybrene. Twenty-four hours later, cells were infected with vector control, FRP1, or DKK1 possessing either a puromycin or geneticin marker. Cells were selected for two weeks by addition of puromycin (0.5–2 μg/ml) or geneticin (750 μg/ml) to the growth media. In some cases, subconfluent cultures were transfected using Fugene (Roche) according to the manufacturer's instructions.

### RT-PCR

Total RNAs were extracted using Triazol (Invitrogen) and were reverse transcribed using the Superscript II Reverse Trancriptase (Invitrogen). 10 μl of a 100μl cDNA reaction was utilized as template for amplification with the following primers specific for each Wnt. For human *Wnt-2* forward: 5′-TGGCTC CCTCTGCTCTTGACC-3′, and reverse: 5′-AGTCAATGTTATCACTGCAGC-3′; for human *Wnt-3* forward: 5′-GAAGGCTGGAAGTGGGGCGGCT-3′, and reverse: 5′-GTCTCCACCCAGCCTCGGGACTCA-3′; for human *Wnt-3a* forward: 5′-GGATACTTCTTACTCCTCTGCAG, and reverse: 5′-AATGGCGTG GACAAAGGCCGACT. Expression of other Wnt family members was analyzed utilizing the Human WNT gene family multigene-12 RT-PCR profiling kit (SuperArray).

### GST-E-cadherin binding assay and immunoblot analysis

The GST-E-cadherin binding assay has been previously described (Bafico et al., 1998). Uncomplexed β-catenin present in 1 mg of total cell lysate was subjected to SDS-PAGE and detected using a monoclonal antibody to β-catenin (Transduction Laboratories). Unphosphorylated β-catenin was detected with a monoclonal antibody specific for β-catenin dephosphorylated at residues 27–37 (Alexis). FRP1-HA and DKK1-HA were detected with an anti-HA monoclonal antibody (Hybridoma Center, Mount Sinai School of Medicine, New York).

### Apoptosis assay

Subconfluent cultures were treated with increasing amounts of tert-butyl hydroperoxide (tBH) for 2 hr. Twenty-four hours later, cells were washed in PBS, trypsinized, and incubated with Annexin and PI using the Annexin-V-Fluos Staining kit (Roche). Fluorescent stained cells were subjected to FACS (Beckton Dickinson FACScan) using Cell Quest 3.2 software (Beckton Dickinson) for acquisition and analysis.

### Luciferase reporter assays

Cells plated at $3 \times 10^5$ per well in 6-well plates were cotransfected with 1 μg of either the TOP-glow or FOP-glow plasmids (Upstate Biotechnology) and 0.001 μg of the Renilla control plasmid (pRL-CMV) utilizing Fugene (Roche) according to the manufacturer's instructions. After 48 hr, cells were

lysed and analyzed utilizing the Dual Luciferase Reporter Assay system (Promega).

**RNA interference**

siRNAs were constructed in the pSuper expression vector as previously described (Brummelkamp et al., 2002). The 19-nucleotide target sequence for *LRP6* was 5′-CCGCATGGTGATTGATGAA-3′, and for *LRP5* was 5′-CAT GATCGAGTCGTCCAAC-3′. 293 T or PAI cells were transiently transfected using Fugene (Roche) and analyzed after 72 hr.

**Northern blot analysis**

RNAs were extracted using Triazol (Invitrogen), separated by agarose gel electrophoresis, and transferred to a nylon membrane (Hybond, Pharmacia). Probes were labeled by the Random Prime Labeling System method (Amersham Biosciences), and hybridization was performed overnight utilizing the Hybrisol I solution (Serologicals Corporation) according to the manufacturer's instructions. Normalization was performed utilizing a commercial human GADPH control probe (BD Biosciences Clontech).

**Tumorigenicity assays**

Parental and wild-type β-*catenin* allele-containing HCT116 cells were transfected with either vector control or FRP1. Marker-selected mass cultures of each were subcutaneously injected in 6 week old nude mice at $2.5 \times 10^6$ cells per site. Tumor growth was monitored at weekly intervals as previously described (Pierce et al., 1991).

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**References**

Alonso, L., and Fuchs, E. (2003). Stem cells in the skin: Waste not, Wnt not. Genes Dev. *17*, 1189–1200.

Bafico, A., and Aaronson, S.A. (2003). Growth factors and signal transduction in cancer. In Cancer Medicine, 6th edition, D.W. Kufe, R.E. Pollock, R.R. Weichselbaum, R.C. Bast, T.S. Gansler, J.F. Holland, and E. Frei, eds. (Hamilton, Ontario: BC Decker), pp. 53–71.

Bafico, A., Gazit, A., Wu-Morgan, S.S., Yaniv, A., and Aaronson, S.A. (1998). Characterization of Wnt-1 and Wnt-2 induced growth alterations and signaling pathways in NIH3T3 fibroblasts. Oncogene *16*, 2819–2825.

Bafico, A., Gazit, A., Pramila, T., Finch, P.W., Yaniv, A., and Aaronson, S.A. (1999). Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. J. Biol. Chem. *274*, 16180–16187.

Bafico, A., Liu, G., Yaniv, A., Gazit, A., and Aaronson, S.A. (2001). Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. Nat. Cell Biol. *3*, 683–686.

Bejcek, B.E., Li, D.Y., and Deuel, T.F. (1989). Transformation by v-sis occurs by an internal autoactivation mechanism. Science *245*, 1496–1499.

Bienz, M., and Clevers, H. (2000). Linking colorectal cancer to Wnt signaling. Cell *103*, 311–320.

Blasband, A., Schryver, B., and Papkoff, J. (1992). The biochemical properties and transforming potential of human Wnt-2 are similar to Wnt-1. Oncogene *7*, 153–161.

Blume-Jensen, P., and Hunter, T. (2001). Oncogenic kinase signalling. Nature *411*, 355–365.

Brown, A.M. (2001). Wnt signaling in breast cancer: Have we come full circle? Breast Cancer Res. *3*, 351–355.

Brummelkamp, T.R., Bernards, R., and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. Science *296*, 550–553.

Bui, T.D., Rankin, J., Smith, K., Huguet, E.L., Ruben, S., Strachan, T., Harris, A.L., and Lindsay, S. (1997). A novel human Wnt gene, WNT10B, maps to 12q13 and is expressed in human breast carcinomas. Oncogene *14*, 1249–1253.

Cadigan, K.M., and Nusse, R. (1997). Wnt signaling: A common theme in animal development. Genes Dev. *11*, 3286–3305.

Caldwell, G.M., Jones, C., Gensberg, K., Jan, S., Hardy, R.G., Byrd, P., Chughtai, S., Wallis, Y., Matthews, G.M., and Morton, D.G. (2004). The Wnt antagonist sFRP1 in colorectal tumorigenesis. Cancer Res. *64*, 883–888.

Chan, T.A., Wang, Z., Dang, L.H., Vogelstein, B., and Kinzler, K.W. (2002). Targeted inactivation of CTNNB1 reveals unexpected effects of β-catenin mutation. Proc. Natl. Acad. Sci. USA *99*, 8265–8270.

Chen, S., Guttridge, D.C., You, Z., Zhang, Z., Fribley, A., Mayo, M.W., Kitajewski, J., and Wang, C.Y. (2001). Wnt-1 signaling inhibits apoptosis by activating β-catenin/T cell factor-mediated transcription. J. Cell Biol. *152*, 87–96.

Dale, T.C., Weber-Hall, S.J., Smith, K., Huguet, E.L., Jayatilake, H., Gusterson, B.A., Shuttleworth, G., O'Hare, M., and Harris, A.L. (1996). Compartment switching of WNT-2 expression in human breast tumors. Cancer Res. *56*, 4320–4323.

Doolittle, R.F., Hunkapiller, M.W., Hood, L.E., Devare, S.G., Robbins, K.C., Aaronson, S.A., and Antoniades, H.N. (1983). Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science *221*, 275–277.

Fedi, P., Bafico, A., Nieto Soria, A., Burgess, W.H., Miki, T., Bottaro, D.P., Kraus, M.H., and Aaronson, S.A. (1999). Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling. J. Biol. Chem. *274*, 19465–19472.

Filali, M., Cheng, N., Abbott, D., Leontiev, V., and Engelhardt, J.F. (2002). Wnt-3A/β-catenin signaling induces transcription from the LEF-1 promoter. J. Biol. Chem. *277*, 33398–33410.

Finch, P.W., He, X., Kelley, M.J., Uren, A., Schaudies, R.P., Popescu, N.C., Rudikoff, S., Aaronson, S.A., Varmus, H.E., and Rubin, J.S. (1997). Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. Proc. Natl. Acad. Sci. USA *94*, 6770–6775.

Giles, R.H., van Es, J.H., and Clevers, H. (2003). Caught up in a Wnt storm: Wnt signaling in cancer. Biochim. Biophys. Acta *1653*, 1–24.

Glinka, A., Wu, W., Delius, H., Monaghan, A.P., Blumenstock, C., and Niehrs, C. (1998). Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. Nature *391*, 357–362.

Going, J.J. (2003). Stages on the way to breast cancer. J. Pathol. *199*, 1–3.

He, T.C., Sparks, A.B., Rago, C., Hermeking, H., Zawel, L., da Costa, L.T., Morin, P.J., Vogelstein, B., and Kinzler, K.W. (1998). Identification of c-MYC as a target of the APC pathway. Science *281*, 1509–1512.

Huang, J.S., Huang, S.S., and Deuel, T.F. (1984). Transforming protein of simian sarcoma virus stimulates autocrine growth of SSV-transformed cells through PDGF cell-surface receptors. Cell *39*, 79–87.

Hudziak, R.M., Lewis, G.D., Winget, M., Fendly, B.M., Shepard, H.M., and Ullrich, A. (1989). p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol. Cell. Biol. *9*, 1165–1172.

Huguet, E.L., McMahon, J.A., McMahon, A.P., Bicknell, R., and Harris, A.L. (1994). Differential expression of human Wnt genes *2*, *3*, *4*, and *7B* in human

breast cell lines and normal and disease states of human breast tissue. Cancer Res. *54*, 2615–2621.

Hulsken, J., Birchmeier, W., and Behrens, J. (1994). E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. J. Cell Biol. *127*, 2061–2069.

Kawano, Y., and Kypta, R. (2003). Secreted antagonists of the Wnt signalling pathway. J. Cell Sci. *116*, 2627–2634.

Keating, M.T., and Williams, L.T. (1988). Autocrine stimulation of intracellular PDGF receptors in v-sis-transformed cells. Science *239*, 914–916.

Kuhnert, F., Davis, C.R., Wang, H.T., Chu, P., Lee, M., Yuan, J., Nusse, R., and Kuo, C.J. (2004). Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. Proc. Natl. Acad. Sci. USA *101*, 266–271.

Lee, B.A., and Donoghue, D.J. (1992). Intracellular retention of membrane-anchored v-sis protein abrogates autocrine signal transduction. J. Cell Biol. *118*, 1057–1070.

Lepourcelet, M., Chen, Y.N., France, D.S., Wang, H., Crews, P., Petersen, F., Bruseo, C., Wood, A.W., and Shivdasani, R.A. (2004). Small-molecule antagonists of the oncogenic Tcf/β-catenin protein complex. Cancer Cell *5*, 91–102.

Leyns, L., Bouwmeester, T., Kim, S.H., Piccolo, S., and De Robertis, E.M. (1997). Frzb-1 is a secreted antagonist of Wnt signaling expressed in the Spemann organizer. Cell *88*, 747–756.

Li, Y., Welm, B., Podsypanina, K., Huang, S., Chamorro, M., Zhang, X., Rowlands, T., Egeblad, M., Cowin, P., Werb, Z., et al. (2003). Evidence that transgenes encoding components of the Wnt signaling pathway preferentially induce mammary cancers from progenitor cells. Proc. Natl. Acad. Sci. USA *100*, 15853–15858.

Lin, K., Wang, S., Julius, M.A., Kitajewski, J., Moos, M., Jr., and Luyten, F.P. (1997). The cysteine-rich frizzled domain of Frzb-1 is required and sufficient for modulation of Wnt signaling. Proc. Natl. Acad. Sci. USA *94*, 11196–11200.

Lin, S.Y., Xia, W., Wang, J.C., Kwong, K.Y., Spohn, B., Wen, Y., Pestell, R.G., and Hung, M.C. (2000). β-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc. Natl. Acad. Sci. USA *97*, 4262–4266.

Liu, G., Bafico, A., Harris, V.K., and Aaronson, S.A. (2003). A novel mechanism for Wnt activation of canonical signaling through the LRP6 receptor. Mol. Cell. Biol. *23*, 5825–5835.

Liu, B.Y., McDermott, S.P., Khwaja, S.S., and Alexander, C.M. (2004). The transforming activity of Wnt effectors correlates with their ability to induce the accumulation of mammary progenitor cells. Proc. Natl. Acad. Sci. USA *101*, 4158–4163.

Longo, K.A., Kennell, J.A., Ochocinska, M.J., Ross, S.E., Wright, W.S., and MacDougald, O.A. (2002). Wnt signaling protects 3T3–L1 preadipocytes from apoptosis through induction of insulin-like growth factors. J. Biol. Chem. *277*, 38239–38244.

Macip, S., Igarashi, M., Berggren, P., Yu, J., Lee, S.W., and Aaronson, S.A. (2003). Influence of induced reactive oxygen species in p53-mediated cell fate decisions. Mol. Cell. Biol. *23*, 8576–8585.

Mao, B., Wu, W., Li, Y., Hoppe, D., Stannek, P., Glinka, A., and Niehrs, C. (2001). LDL-receptor-related protein 6 is a receptor for Dickkopf proteins. Nature *411*, 321–325.

Mao, B., Wu, W., Davidson, G., Marhold, J., Li, M., Mechler, B.M., Delius, H., Hoppe, D., Stannek, P., Walter, C., et al. (2002). Kremen proteins are Dickkopf receptors that regulate Wnt/β-catenin signalling. Nature *417*, 664–667.

Morin, P.J., Sparks, A.B., Korinek, V., Barker, N., Clevers, H., Vogelstein, B., and Kinzler, K.W. (1997). Activation of β-catenin-Tcf signaling in colon cancer by mutations in β-catenin or APC. Science *275*, 1787–1790.

Myers, C., Cooper, M., Stein, C., LaRocca, R., Walther, M.M., Weiss, G., Choyke, P., Dawson, N., Steinberg, S., Uhrich, M.M., et al. (1992). Suramin: A novel growth factor antagonist with activity in hormone-refractory metastatic prostate cancer. J. Clin. Oncol. *10*, 881–889.

Nusse, R., and Varmus, H.E. (1992). Wnt genes. Cell *69*, 1073–1087.

Orford, K., Orford, C.C., and Byers, S.W. (1999). Exogenous expression of β-catenin regulates contact inhibition, anchorage-independent growth, anoikis, and radiation-induced cell cycle arrest. J. Cell Biol. *146*, 855–868.

Palacios, J., and Gamallo, C. (1998). Mutations in the β-catenin gene (CTNNB1) in endometrioid ovarian carcinomas. Cancer Res. *58*, 1344–1347.

Pierce, J.H., Arnstein, P., DiMarco, E., Artrip, J., Kraus, M.H., Lonardo, F., Di Fiore, P.P., and Aaronson, S.A. (1991). Oncogenic potential of erbB-2 in human mammary epithelial cells. Oncogene *6*, 1189–1194.

Pinson, K.I., Brennan, J., Monkley, S., Avery, B.J., and Skarnes, W.C. (2000). An LDL-receptor-related protein mediates Wnt signalling in mice. Nature *407*, 535–538.

Pinto, D., Gregorieff, A., Begthel, H., and Clevers, H. (2003). Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. Genes Dev. *17*, 1709–1713.

Polakis, P. (2000). Wnt signaling and cancer. Genes Dev. *14*, 1837–1851.

Polesskaya, A., Seale, P., and Rudnicki, M.A. (2003). Wnt signaling induces the myogenic specification of resident CD45+ adult stem cells during muscle regeneration. Cell *113*, 841–852.

Rattner, A., Hsieh, J.C., Smallwood, P.M., Gilbert, D.J., Copeland, N.G., Jenkins, N.A., and Nathans, J. (1997). A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. Proc. Natl. Acad. Sci. USA *94*, 2859–2863.

Reya, T., Duncan, A.W., Ailles, L., Domen, J., Scherer, D.C., Willert, K., Hintz, L., Nusse, R., and Weissman, I.L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409–414.

Rockman, S.P., Currie, S.A., Ciavarella, M., Vincan, E., Dow, C., Thomas, R.J., and Phillips, W.A. (2001). Id2 is a target of the β-catenin/T cell factor pathway in colon carcinoma. J. Biol. Chem. *276*, 45113–45119.

Rowlands, T.M., Pechenkina, I.V., Hatsell, S.J., Pestell, R.G., and Cowin, P. (2003). Dissecting the roles of β-catenin and cyclin D1 during mammary development and neoplasia. Proc. Natl. Acad. Sci. USA *100*, 11400–11405.

Sagae, S., Kobayashi, K., Nishioka, Y., Sugimura, M., Ishioka, S., Nagata, M., Terasawa, K., Tokino, T., and Kudo, R. (1999). Mutational analysis of β-catenin gene in Japanese ovarian carcinomas: Frequent mutations in endometrioid carcinomas. Jpn. J. Cancer Res. *90*, 510–515.

Sekine, S., Shibata, T., Sakamoto, M., and Hirohashi, S. (2002). Target disruption of the mutant β-catenin gene in colon cancer cell line HCT116: preservation of its malignant phenotype. Oncogene *21*, 5906–5911.

Semenov, M.V., Tamai, K., Brott, B.K., Kuhl, M., Sokol, S., and He, X. (2001). Head inducer Dickkopf-1 is a ligand for Wnt coreceptor LRP6. Curr. Biol. *11*, 951–961.

Shimizu, H., Julius, M.A., Giarre, M., Zheng, Z., Brown, A.M., and Kitajewski, J. (1997). Transformation by Wnt family proteins correlates with regulation of β-catenin. Cell Growth Differ. *8*, 1349–1358.

Shtutman, M., Zhurinsky, J., Simcha, I., Albanese, C., D'Amico, M., Pestell, R., and Ben-Ze'ev, A. (1999). The cyclin D1 gene is a target of the β-catenin/LEF-1 pathway. Proc. Natl. Acad. Sci. USA *96*, 5522–5527.

Siu, I.M., Robinson, D.R., Schwartz, S., Kung, H.J., Pretlow, T.G., Petersen, R.B., and Pretlow, T.P. (1999). The identification of monoclonality in human aberrant crypt foci. Cancer Res. *59*, 63–66.

Slamon, D.J., Leyland-Jones, B., Shak, S., Fuchs, H., Paton, V., Bajamonde, A., Fleming, T., Eiermann, W., Wolter, J., Pegram, M., et al. (2001). Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N. Engl. J. Med. *344*, 783–792.

Staal, F.J., Noort Mv, M., Strous, G.J., and Clevers, H.C. (2002). Wnt signals are transmitted through N-terminally dephosphorylated β-catenin. EMBO Rep. *3*, 63–68.

Stampfer, M.R., and Bartley, J.C. (1985). Induction of transformation and

continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc. Natl. Acad. Sci. USA *82*, 2394–2398.

Stingl, J., Eaves, C.J., Zandieh, I., and Emerman, J.T. (2001). Characterization of bipotent mammary epithelial progenitor cells in normal adult human breast tissue. Breast Cancer Res. Treat. *67*, 93–109.

Sugrue, M.M., Shin, D.Y., Lee, S.W., and Aaronson, S.A. (1997). Wild-type p53 triggers a rapid senescence program in human tumor cells lacking functional p53. Proc. Natl. Acad. Sci. USA *94*, 9648–9653.

Suzuki, H., Gabrielson, E., Chen, W., Anbazhagan, R., van Engeland, M., Weijenberg, M.P., Herman, J.G., and Baylin, S.B. (2002). A genomic screen for genes upregulated by demethylation and histone deacetylase inhibition in human colorectal cancer. Nat. Genet. *31*, 141–149.

Suzuki, H., Watkins, D.N., Jair, K.W., Schuebel, K.E., Markowitz, S.D., Dong Chen, W., Pretlow, T.P., Yang, B., Akiyama, Y., Van Engeland, M., et al. (2004). Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. Nat. Genet. *36*, 417–422.

Tetsu, O., and McCormick, F. (1999). β-catenin regulates expression of cyclin D1 in colon carcinoma cells. Nature *398*, 422–426.

van de Wetering, M., de Lau, W., and Clevers, H. (2002a). WNT signaling and lymphocyte development. Cell *109* (*Suppl*), S13–S19.

van de Wetering, M., Sancho, E., Verweij, C., de Lau, W., Oving, I., Hurlstone, A., van der Horn, K., Batlle, E., Coudreuse, D., Haramis, A.P., et al. (2002b). The β-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell *111*, 241–250.

van Noort, M., Meeldijk, J., van der Zee, R., Destree, O., and Clevers, H. (2002). Wnt signaling controls the phosphorylation status of β-catenin. J. Biol. Chem. *277*, 17901–17905.

Wang, S., Krinks, M., Lin, K., Luyten, F.P., and Moos, M., Jr. (1997). Frzb, a secreted protein expressed in the Spemann organizer, binds and inhibits Wnt-8. Cell *88*, 757–766.

Waterfield, M.D., Scrace, G.T., Whittle, N., Stroobant, P., Johnsson, A., Wasteson, A., Westermark, B., Heldin, C.H., Huang, J.S., and Deuel, T.F. (1983). Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature *304*, 35–39.

Wehrli, M., Dougan, S.T., Caldwell, K., O'Keefe, L., Schwartz, S., Vaizel-Ohayon, D., Schejter, E., Tomlinson, A., and DiNardo, S. (2000). arrow encodes an LDL-receptor-related protein essential for Wingless signalling. Nature *407*, 527–530.

Wiley, H.S., Woolf, M.F., Opresko, L.K., Burke, P.M., Will, B., Morgan, J.R., and Lauffenburger, D.A. (1998). Removal of the membrane-anchoring domain of epidermal growth factor leads to intracrine signaling and disruption of mammary epithelial cell organization. J. Cell Biol. *143*, 1317–1328.

Wong, G.T., Gavin, B.J., and McMahon, A.P. (1994). Differential transformation of mammary epithelial cells by Wnt genes. Mol. Cell. Biol. *14*, 6278–6286.

Wu, R., Zhai, Y., Fearon, E.R., and Cho, K.R. (2001). Diverse mechanisms of β-catenin deregulation in ovarian endometrioid adenocarcinomas. Cancer Res. *61*, 8247–8255.

You, Z., Saims, D., Chen, S., Zhang, Z., Guttridge, D.C., Guan, K.L., MacDougald, O.A., Brown, A.M., Evan, G., Kitajewski, J., and Wang, C.Y. (2002). Wnt signaling promotes oncogenic transformation by inhibiting c-Myc-induced apoptosis. J. Cell Biol. *157*, 429–440.

Yu, Q., Geng, Y., and Sicinski, P. (2001). Specific protection against breast cancers by cyclin D1 ablation. Nature *411*, 1017–1021.