Activation of AXIN2 Expression by β-Catenin-T Cell Factor
A FEEDBACK REPRESSOR PATHWAY REGULATING Wnt SIGNALING*

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The Wnt pathway regulates cell fate, proliferation, and apoptosis, and defects in the pathway play a key role in many cancers. Although Wnts act to stabilize β-catenin levels in the cytosol and nucleus, a multiprotein complex containing adenomatous polyposis coli, glycogen synthase kinase 3β, and Axin1 or its homolog Axin2/Axin1/conductin promotes β-catenin phosphorylation and subsequent proteasomal degradation. We found that the rat Axil gene was strongly induced upon neoplastic transformation of RK3E cells by mutant β-catenin or γ-catenin or after ligand-induced activation of a β-catenin-estrogen receptor fusion protein. Expression of Wnt1 in murine breast epithelial cells activated the conductin gene, and human cancers with defective β-catenin regulation had elevated AXIN2 gene and protein expression. Expression of AXIN2/Axil was strongly repressed in cancer cells by restoration of wild type adenomatous polyposis coli function or expression. AXIN2/Axil expression was strongly repressed in cancer cells by restoration of wild type adenomatous polyposis coli function or expression of a dominant negative form of T cell factor (TCF)-4. TCF binding sites in the AXIN2 promoter played a key role in the ability of β-catenin to activate AXIN2 transcription. In contrast to AXIN2/Axil, expression of human or rat Axin1 homologs was nominally affected by β-catenin-TCF. Because Axin2 can inhibit β-catenin abundance and function, the data implicate AXIN2 in a negative feedback pathway regulating Wnt signaling. Additionally, although Axin1 and Axin2 have been thought to have comparable functions, the observation that Wnt pathway activation elevates AXIN2 but not AXIN1 expression suggests that there may be potentially significant functional differences between the two proteins.

The Wnt signaling pathway plays an important role in cellular proliferation, differentiation, and morphogenesis, and control of β-catenin stability is central to Wnt signaling (1–6). In brief, Wnts activate transmembrane frizzled receptors and the disheveled protein, leading to inhibition of glycogen synthase kinase 3β (GSK3β)1 activity. Typically, GSK3β, when active and present in a multiprotein complex containing the APC (adenomatous polyposis coli) tumor suppressor and Axin1 and/or Axin2 (also known as Axil or conductin), can phosphorylate specific serine and/or threonine residues near the β-catenin N terminus (6–10). The phosphorylated forms of β-catenin bind to the F box protein β-TrCP, a subunit of the SCF-type E3 ubiquitin ligase complex, resulting in ubiquitination of β-catenin and its ultimate degradation by the proteasome (5, 6, 11–14). Wnt pathway activation inhibits GSK3β activity, causing β-catenin to accumulate in the cytoplasm and nucleus, where it can bind to members of the TCF (T cell factor)/LEF (lymphoid enhancer family) transcription factor family (referred to hereafter collectively as TCFs) (1–5). In the nucleus, TCFs mediate sequence-specific DNA binding, and β-catenin, via its interaction with TCFs, affects transcription of genes with TCF binding sites in their regulatory regions. Thus far, it appears that β-catenin generally activates TCF-regulated genes and that β-catenin-TCF target genes include c-myc, cyclin D1 (CCND1), matrilysin/MMP-7, Tcf-1, PPARδ, PEA3, EN1, c-ETS2, c-myb, and c-kit (15–23).

Defects that interfere with β-catenin regulation have been reported in various human cancers. In a subset of many different cancer types, mutations at or near the serine and threonine residues in the β-catenin N-terminal domain alter its ability to be phosphorylated by GSK3β. In other cancers, particularly colorectal cancers, inactivation of the APC tumor suppressor gene appears to be the predominant mechanism leading to β-catenin deregulation (4, 5, 24). In yet other cancers, mutations in the genes encoding one of the two Axin proteins have been reported, including the AXIN1 gene in hepatocellular carcinomas and medulloblastomas (25, 26) and the AXIN2 gene in a small fraction of colorectal cancers lacking APC or β-catenin mutations (27). A prime consequence of the mutational defects in β-catenin regulation is constitutive activation of downstream β-catenin-TCF-regulated target genes, particularly genes with major effects on cell growth regulation and tumorigenesis, such as c-myc, CCND1, and MMP-7 (4, 5).

In an effort to understand better the effects of Wnt-β-catenin-TCF pathway activation in cancer cells, we undertook studies to identify novel β-catenin-TCF-regulated target genes. We used oligonucleotide microarrays to identify transcripts with elevated expression after neoplastic transformation of the rat E1A-immortalized RK3E cell line by mutant β-catenin or γ-catenin or after ligand-induced activation of a β-catenin-estrogen receptor (ER) fusion protein. We found that expression of the rat Axil gene was strongly induced in the RK3E cell line in all three of these settings. Further studies established that the mouse and human homologs of Axil, known as conductin and AXIN2, respectively, were consistently induced by Wnt pathway activation. TCF proteins played a key role in AXIN2...
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induction. Unlike AXIN2, AXIN1 was not found to be a β-cate-
nin-TCF-regulated gene. Prior studies have shown that the Axin1 and Axin2 proteins have roughly 45% amino acid identity and essentially identical functions in regulating β-catenin levels (7–10, 28). In addition to showing that AXIN2 functions in a feedback repression pathway regulating Wnt signaling, our findings on the differential effects of Wnt pathway activation on AXIN2 versus AXIN1 expression suggest that potentially significant functional differences may exist between their pro-
tein products.

EXPERIMENTAL PROCEDURES

Plasmids—Expression vectors for wild type and mutant (codon 33 substitution of tyrosine for serine, S33Y) forms of β-catenin and domin-
ant negative Tcf-4 (Tcf-4ΔN31) have been described previously (29). The pBabe-S33Y-ER-puro expression vector encoding a chimeric β-cate-
nin-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 BamHI and EcoRI sites of the retroviral plasmid pBabe-puro (30). The reporter constructs pTOP-
FLASH, which contains three copies of an optimal TCF binding motif (CCTTTGATTC), and pFOPFLASH, which contains three copies of a mutant TCF motif (CCTTTGCC), have been described previously (31). Plasmid pCH110 (Amersham Biosciences) contains a functional lacZ gene cloned downstream from a cytomegalovirus early region promoter-
enhancer element. The Axin2pcDNA3.1mycHIS−β expression vector was a kind gift from Wanguo Liu (Mayo Clinic, Rochester, MN) (27).

DNA fragments containing human AXIN2 promoter sequences cloned upstream from a luciferase reporter gene were obtained by PCR ampli-
fication of genomic DNA, using primers generated from AXIN2 se-
quences in GenBank (accession no. AC004855). AXIN2 genomic DNA fragments were subcloned upstream from the luciferase reporter gene in the pGLOBasic reporter vector (Promega, Madison, WI), using the KpnI and NheI sites. The reporter gene vector AX2/1078WT/RLuc con-
tains AXIN2 sequences from −1078 to +5 relative to the presumed transcription start site, and the vector AX2/181WT/RLuc contains AXIN2 sequences from −181 to +5. The forward primer for generating the AX1078WT/RLuc vector was 5′-CCGTTTACGCCTACTCCTCT-
TAG-3′, and the forward primer for the AX181WT/RLuc vector was 5′-CAGCGCTTGATACCTGACTGAGC-3′. The reverse primer for gener-
generating both vectors was 5′-CAAGTCAGGCGGCTTACAGC-3′. Mu-
tations in a presumptive TCF DNA binding site at bp −108 to −102 were designed in vitro via a standard PCR-based mutagenesis strategy,
activating the S33Y-ER fusion protein, the RK3E/S33Y-ER cells were treated with medium supplemented with 0.5 μM 4-hydroxytamoxifen (4-OH-T) (Sigma), made from a stock concentration of 100 μM 4-OH-T in 100% ethanol. To inhibit new protein synthesis in RK3E/33Y-ER cells, the medium was supplemented with cycloheximide (Sigma) at a concentration of 1 μg/ml. To assess the effects of total β-catenin on AXIN2 and AXIN1 gene expression, a retroviral TCF-4ΔN31 expres-

DNA Array Expression Analysis—Trizol (Invitrogen) extraction and purifica-
tion with the RNeasy Cleanup Kit (Qiagen, Chatsworth, CA) was used to prepare total RNA from five samples: parental RK3E cells; RK3E/33Y-ER cells either mock (ethanol)-treated or 4-OH-T-treated for 24 h; a pool of equal masses of RNA from seven cloned RK3E lines transformed neoplastically by mutant β-catenin (29); and a pool of equal masses of RNA from five cloned RK3E lines transformed neoplastically by wild type β-catenin (35). Genomic DNAs from these cell lines were carried out with commercial high density oligonucleotide arrays (Affymetrix, Santa Clara, CA), using protocols and methods developed by the suppliers. Arrays were scanned using the GeneArray scanner (Affymetrix), and image analysis was performed with GeneChip 4.0 software (Affymetrix), which stores the results for each feature in .CEL files. Each RG_U34A chip consists of 534 × 534 probes (24 × 24 μm each) that are 25-base long single-stranded DNA sequences. There are typically 16 pairs of features (probe pairs) for each of the transcripts (probe sets) and a total of 8,799 probe sets. Half of the features are com-
plementary to a specific sequence (perfect match = PM features), the other half have an identical match except a central base has been altered (mismatch = MM features). We have developed software to read .CEL files and perform some processing of the data, available at dot.ped.med.uic.edu:2000/ourimage/pub/shared/AFmethods.html. The chip for the parental RK3E sample was selected as a standard. Probe sets for which PM-MM < 1.0 were excluded from the analysis. One-sided signed rank tests of the PM-MM values for each probe set on both vectors were obtained to help judge whether transcripts were detectable. The average intensity for each probe set was normalized as the average of PM-MM differences, after trimming away the 25% highest and lowest differences. A set of 3,699 reference probe sets was selected for use in normalization, these being the probe sets that gave p < 0.05 for all five chips for the test of detectability. A normalization factor for each chip was obtained using the reference probe sets by computing the antilogarithm of the mean log ratios of the average intensities for the selected chip divided by the standard. The log ratios were then corrected with this factor to give the final normalized intensities for the probe sets. When computing fold change indices, we replaced intensities less than 10 by 10 before forming ratios to avoid negative or spuriously large fold change numbers.

Northern Blot Analysis—Total RNA was extracted from cells with Trizol, and Northern blot analysis was performed. Approximately 15–20 μg of total RNA was separated on a 1.2% formaldehyde–agarose gel and transferred to Zeta-Probe GT membranes (Bio-Rad) by capillary action. CIDNA probes to detect rat Axin1, mouse Apc, rat Apc, and human AXIN1 expression were generated by RT-PCR, using primers derived from sequences in GenBank. The probe to detect AXIN2 was generated by PCR using the Apc2pcDNA3myc.3 plasmid (provided by W. Liu; Mayo Clinic). The sequences of all PCR products were confirmed by automated sequencing. All probes were random labeled with [α-32P]dCTP using Rediprime (Invitrogen) and hybridized to the membrane with RapidHyb Buffer (Invitrogen) according to the manu-
facturer’s protocol. All Northern blots were stripped and hybridized to a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe to control for RNA loading and transfer efficiency.

Western Blot Analysis—Whole cell lysates were prepared in radiom-
ochrome precipitation assay buffer (Tri-free buffered saline TBS, 0.5% deoxy-
cholic acid, 0.1% SDS, and 1% Nonidet P-40 with complete protease inhibitors (Roche Molecular Biochemicals)). Protein concentration was determined by the bicinchoninic acid assay (Pierce Biochemicals), and 50 μg of total protein from each sample was separated on 10% SDS-
polyacrylamide gels. Proteins were transferred to Immobilon P mem-
brenes (Millipore, Bedford, MA) by semidry electrol blotting. Immuno-

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The pTOPFLASH vector to the pFOPFLASH vector. All luciferase transcriptional activity was measured as the ratio of luciferase activity from sates were collected in 1/100.Cells were plated in 35-mm six-well plates 12–24 h before transfection.

\[ \text{ABC kit; Vector Laboratories, Burlingame, CA). Immuno-} \]
\[ \text{tastain ABC kit; Vector Laboratories, Burlingame, CA). Immuno-} \]
\[ \text{nant antibody complexes were detected with the avidin-biotin peroxidase} \]
\[ \text{overnight at 4°C for 1 h. Sections were then incubated with the anti-conductin (M-20)} \]
\[ \text{phosphorylation consensus sites to activate γ-catenin's transforming potential} \]
\[ \text{and mean ± S.D. values were determined.} \]

RESULTS

Induction of Axil Expression by β- or γ-Catenin Deregulation or Ligand-induced Activation of a β-Catenin-ER Fusion Protein—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, will promote neoplastic transformation of RK3E cells, a rat E1A-immortalized epithelial cell line (29). 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 GSK3β phosphorylation.

Axil gene expression were seen in 8 of 10 Independent β-catenin-transformed RK3E lines might simply reflect the clonal origin of the transformed line under study, for the array analysis, we pooled equal masses of RNA from seven independent γ-catenin-transformed lines. In addition, because some changes in gene expression after transformation of RK3E cells by β- or γ-catenin might be the result of alterations in signaling pathways unrelated to catenin-TCF deregulation, we also assessed the consequences of transient activation of the Axil gene expression. Transient activation of β-catenin in RK3E cells was achieved by treatment of an RK3E cell line expressing a chimeric β-catenin-ER fusion protein (RK3E/S33Y-ER) with the ligand 4-OH-T for 24 h. For our studies, we used the Affymetrix U34A rat GeneChip array, which contains roughly 8,000 known genes and expressed sequence tags. By comparing gene expression in parental RK3E cells and mock-treated RK3E/S33Y-ER cells (i.e. the two control cell populations) with gene expression in β- and γ-catenin-transformed RK3E and 4-OH-T-treated RK3E/S33Y-ER cells, we identified only 14 genes predicted to have greater than 2-fold increases in expression over control levels after catenin-TCF activation.

Northern blotting was used to assess the 14 candidate genes identified by the array analysis, and the most promising data were obtained for the rat Axil gene. Compared with parental RK3E cell lines or RK3E lines transformed by mutated K-ras or Gli1, marked increases in Axil expression were seen in 8 of 10 independent β-catenin-transformed RK3E lines (Fig. 1A) and all eight γ-catenin-transformed RK3E lines studied (Fig. 1C). In contrast to the strong induction of Axil, expression of the rat Axin1 gene was not altered in RK3E lines transformed by β-catenin (Fig. 1B). Confirmation that induction of Axil gene expression resulted in changes in expression of the Axil protein was documented in selected β-catenin-transformed RK3E lines (Fig. 1D). Rapid induction of Axil expression was also seen in the RK3E/S33Y-ER cell line after treatment with the 4-OH-T ligand (Fig. 2A). The observation that the protein synthesis inhibitor cycloheximide did not block 4-OH-T-mediated induction of Axil expression in RK3E/S33Y-ER cells (Fig. 2A) indicates Axil is very likely to be a gene activated directly by β-catenin accumulation in the nucleus. Consistent with the view that Axil is induced by β-catenin interaction with TCF transcription factors, expression of a dominant negative form of

PCH110. For reporter gene assays with AXIN2 promoter constructs, DLD1 cells were cotransfected with 1 μg of AX2/1078WT/Luc or AX2/1078Mut/Luc and 1 μg of pCH110, whereas SW480/Neo or SW480/Tcf4-LN31 cells were cotransfected with 1 μg of AX2/181WT/ Luc or AX2/181Mut/Luc and 1 μg of pCH110. The total mass of trans-
TCF-4 in RK3E cell lines stably transformed by mutant β-catenin inhibited TCF transcriptional activity (Fig. 2B) and Axil expression (Fig. 2C).

Axil Homologs in Mouse and Man Are Downstream Targets of the Wnt Pathway—In an effort to establish that the Wnt pathway regulates expression of Axil or its homologs in other
systems and settings, we analyzed expression of *conductin*, the mouse homolog of Axil, in breast epithelial cells expressing high levels of the Wnt-1 protein. In both RAC311 and C57 cells, Wnt-1 expression was associated with high levels of *conductin* expression (Fig. 3).

As noted above, inactivating mutations in the *APC* gene are common in human colon cancers, and a subset of the 20–25% of colon cancers that lack *APC* mutations have gain-of-function mutations in the β-catenin N terminus (4, 5). Both the inactivating mutations in *APC* and the activating mutations in β-catenin led to β-catenin deregulation and constitutive activation of β-catenin-TCF transcripton. Consistent with the notion that the human AXIN2 gene might also be a target of the Wnt pathway, we found variable but readily detectable expression of AXIN2 in all 12 colon cancer cell lines studied (Fig. 4A and data not shown). To explore further the relationship between β-catenin deregulation and AXIN2 expression in colon cancers, we took advantage of a colon cancer cell line with regulated expression of the wild type *APC* gene. The HT29 colon cancer line has truncating mutations in both of its *APC* alleles. Morin et al. (34) generated a variant HT29 line (HT29/APC) in which, after zinc treatment, expression of an exogenous wild type *APC* protein is induced rapidly to roughly the same level as that of the endogenous truncated *APC* proteins. Using HT29/APC cells and a matched control line (i.e. HT29/β-Gal), we found that AXIN2 expression was strongly down-regulated after APC induction. Zinc treatment of the control HT29/β-Gal cell line had no detectable effect on AXIN2 expression. In contrast to the AXIN2 results, restoration of APC function in HT29 cells had only modest effects on AXIN1 expression. Furthermore, expression of a dominant negative form of TCF-4 in DLD1 and SW480 colon cancer cells strongly inhibited TCF transcriptional activity and AXIN2 expression but had at best minimal effects on AXIN1 expression (Fig. 5).

Nearly all candidate β-catenin-TCF-regulated genes described in the literature have been proposed based on data from *in vitro* and/or animal model studies. Thus far, few studies have evaluated expression of presumptive β-catenin-TCF target genes in primary human tumors that have been characterized thoroughly for mutational defects in β-catenin regulation. We chose to assess AXIN2 expression in primary OEsAs because although OEsAs share similar histological features, only about 30–40% of the lesions have mutational defects affecting β-catenin regulation (36–38). This contrasts with the picture in primary colorectal carcinomas, which almost uniformly carry mutational defects in β-catenin regulation (4, 5). Hence, comparison of gene expression in OEsAs with intact β-catenin regulation versus OEsAs with defective β-catenin regulation should permit a more definitive assessment to be made about the relationship between β-catenin regulatory defects and expression of candidate β-catenin-TCF target genes. Using real time RT-PCR assays to assess AXIN2 expression in a panel of 42 OEsAs characterized previously for β-catenin nuclear localization and mutations in the β-catenin, *APC*, AXIN1, and AXIN2 genes (36), we found that AXIN2 expression was on average roughly 20-fold higher in the OEsAs with β-catenin regulatory defects than in OEsAs with apparently intact β-catenin regulation (Fig. 6). To confirm that induction of AXIN2 gene expression resulted in demonstrable changes in AXIN2 protein expression in primary tumors with β-catenin defects, we performed immunohistochemistry studies on a subset of the OEsAs analyzed in the real time RT-PCR studies. AXIN2 expression was found to be increased in the majority of OEsAs with β-catenin regulatory defects compared with those OEsAs with intact β-catenin regulation (examples in Fig. 7).

**Critical Role of TCF Binding Sites in AXIN2 Proximal Promoter in β-Catenin-mediated Induction**—To establish further the role of TCFs in regulating AXIN2 expression, we examined AXIN2 genomic sequences for candidate TCF binding sites. The only consensic TCF binding site identified in a search of sequences located from −1500 to +500 bp (relative to the presumed transcriptional start site) was found at −108 to −102 (i.e. CTTTGGTC; Fig. 8A). Luciferase reporter gene constructs containing this element as well as reporter gene constructs in which the element was mutated to CTTTGGCC were generated. In DLD1 and SW480 colon cancer cells, we found that mutation of the consensus TCF site in the AXIN2 promoter markedly decreased the activity of a reporter construct containing roughly 1.0 kb of AXIN2 promoter sequence (Fig. 8B and data not shown). Similar results were obtained in DLD1 and SW480 colon cancer cells with wild type and mutant reporter gene constructs containing only 181 bp of AXIN2 promoter sequences (Fig. 8B and data not shown). Moreover, although expression of a dominant negative TCF-4 mutant protein (dnTCF-4) inhibited the activity of the wild type AXIN2 reporter construct, the dnTCF-4 protein had no major effect on the activity of the reporter gene construct harboring mutations in the consensus TCF binding site (Fig. 8B). Interestingly, cotransfection experiments in HEK293, COS, and HeLa cells, in which an expression vector encoding the S33Y mutant β-catenin protein was cotransfected with the AXIN2 reporter gene constructs, revealed that β-catenin was not sufficient on its own for activation of AXIN2 transcription via the proximal TCF element. The differing results in colon cancer versus other cell lines suggest that cellular context, perhaps including the expression of other cellular proteins, may play a role in the ability of β-catenin to activate AXIN2 transcription (see “Discussion”).

The Axin2 Protein Regulates Wild Type but Not Mutant β-Catenin—Prior work has shown the rat Axil and mouse conductin proteins can negatively regulate Wnt signaling perhaps in large part as a result of the ability of Axil/conductin to serve as a “scaffold” for efficient coordination of the interactions of GSK3β, APC, and β-catenin, resulting in the phosphorylation of β-catenin at critical N-terminal sites (10, 28). To confirm that the human Axin2 protein had an analogous function, we assessed its ability to antagonize β-catenin effects on TCF transcription. As shown in Fig. 9, whereas the ability of wild...
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type β-catenin to activate TCF transcription was strongly inhibited by Axin2, the S33Y mutant form of β-catenin was not significantly inhibited by Axin2. These findings indicate that the Axin2 protein is, as expected, a negative regulator of wild type β-catenin and Wnt signaling.

DISCUSSION

The critical role of the Wnt pathway in development has long been appreciated. Nevertheless, only in the recent past has it become abundantly clear that mutations in Wnt pathway components play a prominent role in the pathogenesis of a rather broad array of human cancers (3–5). A principal effect of the loss-of-function mutations in APC 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 deregulation, the ability of β-catenin to complex with TCFs is enhanced and altered transcription of TCF-regulated genes ensues. Thus far, it appears that activation of β-catenin-TCF-regulated target genes is a major consequence of Wnt pathway deregulation in cancer. Candidate β-catenin-TCF target genes described in the literature include c-myc, CCND1, MMP-7, Tcf-1, PPARγ, PEA3, ENC1, c-ETS2, c-mycb, and c-kit (15–23).

In this paper, we have presented a substantial body of data implicating the human AXIN2 gene (and the rat Axil and mouse conductin genes) as a downstream target of the Wnt-β-catenin-TCF pathway. Findings consistent with those we report here on AXIN2/conductin/Axil expression and its regulation by the Wnt pathway were recently published by others (39–41). We initially found that the rat Axil gene was strongly induced upon neoplastic transformation of RK3E cells by mutant β-catenin or γ-catenin or after 4-OH-T-induced activation of a β-catenin-ER fusion protein. In murine breast epithelial cells, we found that overexpression of Wnt1 strongly activated the conductin gene. Human colon cancer cell lines had elevated AXIN2 expression, and restoration of APC function or expression of dominant negative form of TCF-4 in the cells strongly inhibited AXIN2 expression. Primary ovarian carcinomas with defective β-catenin regulation were found to have elevated β-catenin regulation. Consistent with the notion that the AXIN2/Axil/conductin genes are activated directly as a result of binding of the β-catenin-TCF protein complex to regulatory elements within or near the genes, we found that Axil was robustly activated by β-catenin in the absence of new protein synthesis. Use of reporter gene constructs containing proximal promoter sequences from the AXIN2 gene established the ability of β-catenin to activate AXIN2 transcription as well as the key role of TCFs in AXIN2 activation. Although our findings indicate that β-catenin and TCFs play a vital role in the activation of AXIN2 expression in colon and ovarian cancer cells, our observation that the activity of the AXIN2 proximal promoter was not demonstrably affected by β-catenin in several other epithelial
cell types, namely HEK293, COS, and HeLa cells, suggests that the regulation of AXIN2 transcription by β-catenin-TCF may be complex. For example, it is possible that only certain TCF isoforms may bind to and regulate the AXIN2 promoter, and these TCF isoforms display tissue- or cell type-restricted patterns of expression. Alternatively, other transcription factors that bind to specific sites in the AXIN2 promoter may play a key role in cooperating with β-catenin-TCF to activate TCF transcription. Prior studies have suggested that cooperation between β-catenin-TCF and other transcription factors may be important for activation of certain genes, such as the cooperation between β-catenin-TCF and PEA3 in the activation of MMP-7 (42).

In light of prior data in the literature and the data presented here showing that Axin2 can negatively regulate β-catenin function, our findings imply that AXIN2 is a negative feedback regulator of the Wnt pathway. Interestingly, even though the Axin1 and Axin2 proteins appear to have similar functions in negatively regulating β-catenin levels via the ability of the Axins to complex GSK3β, APC, and β-catenin, we obtained no clear-cut evidence that the human AXIN1 gene or its rat homolog rAxin1 was induced by Wnt pathway activation. The differential effects of the Wnt pathway on AXIN1 and AXIN2 suggest that there may be potentially important functional differences between the proteins. For instance, although the two proteins share roughly 45% amino acid identity, they may differ in their ability to interact with other cellular proteins. Thus far, the Axin1 protein has been shown to bind to multiple other proteins besides Wnt pathway factors (i.e. APC, GSK3β, β-catenin, disheveled). These other Axin1-interacting proteins include the following: the mitogen activated protein kinase (MAPK) kinase kinase (MEKK1) protein (43); the GSK3β-binding protein (44); the PR61β and PR64y regulatory subunits of protein phosphatase 2A (45, 46); the low density lipoprotein receptor-related protein-5 (47), which function as a Wnt coreceptor; the transforming growth factor-β pathway transcription factor Smad3 (48); and a novel protein termed Axam (49). Given the apparently large number of Axin1-interacting proteins, if Axin1 expression was strongly induced by Wnt pathway activation, there might be significant effects on many other signaling pathways besides the Wnt pathway. Thus far, it is not clear whether the Axin2 protein binds any or all of these other Axin1-interacting proteins. However, some of the interactions between Axin1 and the non-Wnt pathway-interacting proteins are mediated via regions that are not highly conserved between Axin1 and Axin2. As such, perhaps the differential interactions of Axin1 and Axin2 with certain of the non-Wnt pathway proteins accounts for why Axin2 functions as a major negative feedback regulator of Wnt signaling, and Axin1 does not.

Although bi-allelic inactivation of AXIN1 has been seen in some hepatocellular carcinomas and medulloblastomas (25, 26), indicating that AXIN1 functions as a tumor suppressor gene, bi-allelic inactivation of AXIN2 in cancers has not yet been noted. To date, mutations in the AXIN2 gene appear to be restricted to colon and perhaps other cancers with mismatch repair pathway defects (27, 36). The truncated AXIN2 alleles seen in cancers with mismatch repair defects have been proposed to encode proteins that function in a dominant negative fashion to interfere with β-catenin regulation (27). Because the ability of Axin2 to regulate β-catenin appears to depend upon intact APC function and wild type β-catenin N-terminal sequences, in those cancers with inactivating mutations in APC or oncogenic mutations in β-catenin, elevated Axin2 expression is quite unlikely to have any major inhibitory effect on β-catenin levels and function. Even in cancers with AXIN1 or AXIN2 mutations, because the Axin proteins have been suggested to
observations indicating that the elevated expression of AXIN2 in cancers with Wnt pathway defects is not sufficient to down-regulate β-catenin levels and function, it is possible that Axin2 induction might have other important effects in cancer cells, potentially even growth promoting effects. Further studies of the interactions between Axin2 and other cellular proteins should offer insights into Axin2 function as well as the consequences of its induction by Wnt pathway activation in normal and cancer cells.

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