N-Glycosylation Gene *DPAGT1* Is a Target of the Wnt/β-Catenin Signaling Pathway*

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Protein *N*-glycosylation and the Wnt/β-catenin signaling pathways play critical roles in development and cancer. Although *N*-glycosylation has been shown to influence Wnt signaling through its effects on Wnt ligands, it is unclear whether the Wnt/β-catenin pathway impacts protein *N*-glycosylation. In this study, we show that promoters of the first *N*-glycosylation gene, *DPAGT1*, from Chinese hamster ovary (CHO), Madin-Darby canine kidney (MDCK), and human epidermoid carcinoma (A253) cells contain the T-cell factor/lymphoid enhancer-binding factor (TCF/LEF) consensus sequence. Treatment of cells with a Wnt activator, lithium chloride, up-regulated *DPAGT1* transcript levels that correlated with an increase in the β-catenin abundance. Furthermore, exposure of cells to a Wnt receptor ligand, Wnt3a, resulted in an increase in the *DPAGT1* transcript levels that was abrogated by the Wnt inhibitor, Dickkopf-1. DNA mobility shift assays revealed specific protein complexes at the *DPAGT1* TCF/LEF binding region that were competed off with antibodies to either Tcf3/4 or β-catenin. Chromatin immunoprecipitation analysis confirmed the presence of β-catenin at the *DPAGT1* promoter *in vivo*. In addition, the DPAGT1 TCF/LEF sequence drove the expression of the luciferase reporter gene. Furthermore, up-regulation of DPAGT1 transcripts by Wnt3a led to altered *N*-glycosylation of E-cadherin. Interestingly, the *DPAGT1* TCF/LEF sequence also interacted with γ-catenin, a close homologue of β-catenin, although not in a lithium chloride-dependent manner. Our results provide the first evidence that the Wnt/β-catenin signaling pathway regulates the metabolic pathway of protein *N*-glycosylation by targeting *DPAGT1* expression. Moreover, they suggest the existence of another regulatory mechanism involving the interaction of Tcf with γ-catenin at the *DPAGT1* promoter.

Protein *N*-glycosylation plays critical roles in development and homeostasis by affecting diverse cellular functions (1–4). In addition to the well acknowledged roles of *N*-glycans in protein folding, targeting, and secretion, *N*-glycosylation influences protein-protein interactions and cellular signaling events (1, 2, 5, 7–9). *N*-Glycosylation has been shown to be indispensable for development, and its dysregulation has been linked to various diseases, including cancer (3, 4, 10, 11). Expression of *DPAGT1*, the gene that initiates the synthesis of the lipid-linked oligosaccharide precursor for protein *N*-glycosylation in the endoplasmic reticulum and is a key determinant of the extent of protein *N*-glycosylation (12, 13), is down-regulated with cellular differentiation, suggesting that attenuation of *N*-glycosylation is required for the establishment of a differentiated phenotype (14). Indeed, recent studies have documented that *DPAGT1* is an upstream regulator of *N*-glycosylation status of E-cadherin and that its diminished expression is required for the formation of mature intercellular adhesion complexes (15). Although the importance of *N*-glycosylation in cell and tissue function is well established, little is known about its upstream regulators.

The Wnt signaling pathway has long been known to be critical for a wide variety of developmental processes, including cell fate specification, proliferation, polarity, and migration (16). Although there are at least three Wnt signaling pathways involved in the signal transduction process, the canonical, or β-catenin-dependent, Wnt signaling pathway has been shown to be critical for cell fate decisions, proliferation, and differentiation of diverse cell types (17–19). In the canonical pathway, Wnt proteins bind to Frizzled receptors and trigger intracellular signaling cascades to activate Dishevelled family proteins (20, 21). Activation of Dishevelled inhibits the axin/GSK-3/adenomatous polyposis coli complex and prevents the phosphorylation of β-catenin at the N-terminal serine/threonine residues, leading to stabilization of its cytoplasmic pool. This enables β-catenin to enter the nucleus and interact with the Tcf[2]/Lef family of transcription factors to promote specific gene expression (22). Among the downstream targets of the canonical Wnt pathway are cyclin D1, c-Myc, c-Jun, and matrix metalloproteinases (16, 23, 24). Aberrant activation of the canonical Wnt pathway has been associated with a number of cancers, with inappropriate stabilization of β-catenin playing a key role in tumorigenesis (21). A close homologue of β-catenin, γ-catenin/plakoglobin, also functions as a transcriptional regulator in Wnt signaling and acts as an oncogene, although it appears to have a distinct role in Wnt signaling from that of β-catenin (25, 26).

In addition to its function in the canonical Wnt pathway, β-catenin is a key structural component of cadherin-mediated cell-cell adhesion complexes known as adherens junctions

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[2] The abbreviations used are: Tcf, T-cell factor; AJ, adherens junctions; ChIP, chromatin immunoprecipitation; CHO, Chinese hamster ovary; CM, conditioned medium; LEF, lymphoid enhancer-binding factor; MDCK, Madin-Darby canine kidney; *N*-glycans, asparagine-linked oligosaccharides; EndoH, endoglycosidase H; PNGase, peptide:*N*-glycosidase.

(AJs) (27, 28). Inappropriate phosphorylation of β-catenin at the tyrosine residues has been shown to lead to its dissociation from, and subsequent disassembly of, AJs (29, 30). Such diminished cadherin-mediated adhesion has been linked to cancer development and progression (10, 31, 32). Although γ-catenin has a well established role as a component of desmosomes (33), similar to β-catenin, it also plays a stabilizing role in cadherin junctions. However, little is known about the relationship between its role as an oncogene and a stabilizing component of AJs (34).

Wnts themselves are *N*-glycoproteins, and modification of some Wnts, like Wnt3a, with *N*-glycans has been shown to be required for their function and hence for the activation of the canonical Wnt pathway (35). We now report that the first *N*-glycosylation gene, *DPAGT1*, is a target of the canonical Wnt/β-catenin pathway, indicating that Wnt signaling affects protein *N*-glycosylation. We show that the *DPAGT1* promoter from three different mammalian sources has the TCF/LEF-binding sequence that interacts with Tcf/β-catenin *in vitro* and *in vivo*. *DPAGT1* transcription is activated by a stabilizer of β-catenin, lithium chloride (LiCl), and by the Frizzled ligand, Wnt3a, and is inhibited by the Wnt inhibitor, Dickkopf-1 (Dkk-1). We also show that the TCF/LEF binding region of the *DPAGT1* promoter is functional, because it can drive the expression of the luciferase reporter gene. The *DPAGT1* promoter also binds γ-catenin *in vitro* and *in vivo*, albeit in a manner distinct from that of β-catenin. Our studies provide the first evidence that protein *N*-glycosylation is regulated by the canonical Wnt signaling pathway through the *DPAGT1* gene.

EXPERIMENTAL PROCEDURES

Reagents—Monoclonal antibodies to human E-cadherin, β-catenin, γ-catenin, and IgG isotype controls were obtained from BD Transduction Laboratories. Monoclonal antibody to Tcf3/4 was from Exalpha Biologicals. Polyclonal antibody to myosin II heavy chain isoform B was from Covance, and monoclonal antibody to actin (pan Ab-5, clone ACTN05) was purchased from NeoMarkers. Human, canine, and hamster biotin-derivatized DNA probes spanning the TCF binding region of the respective *DPAGT1* promoters were prepared commercially (Integrated DNA Technologies). LiCl was purchased from Sigma. Rhodamine-phalloidin was obtained from Molecular Probes. Secondary antibodies included goat anti-mouse or anti-rabbit IgG derivatized with fluorescein isothiocyanate (FITC) (Molecular Probes).

Cell Culture and Preparation of Nuclear Extracts—MDCK, CHO, and A253 cells were from American Type Culture Collection and grown in McCoy's 5A, F-12K, and DMEM media, respectively, containing 10% FBS and 1% penicillin/streptomycin. In some cases, cells were treated with either 25 mM lithium chloride, Wnt3a (1 μg/ml), or Wnt antagonist Dkk-1 (1 μg/ml) for 24 h prior to isolation of RNA. To determine the effects of Wnt3a on the expression of *DPAGT1*, *ALG1*, and *MGAT5* in MDCK cells, cells (passage 5) were grown to 80–90% confluence, serum-starved for 24 h, and then grown in the presence of 50% conditioned medium isolated from either L-mouse fibroblasts or L-mouse fibroblasts stably transfected with Wnt3a (ATCC) for 24 h. Total cellular RNA was extracted, reverse-

transcribed, and quantitated using real time PCR. For studies of *N*-glycosylation of E-cadherin, MDCK cells were serum-deprived for 24 h and then grown in the presence of either 50% conditioned medium or 50% conditioned medium containing Wnt3a for 72 h. For the preparation of cell lysates, cells were extracted with 600 μl of ice-cold RIPA buffer (10 mM Tris, 150 mM NaCl, and 1% Triton X-100) containing 25 μg/ml soybean trypsin inhibitor, 100 μM benzamidine, 5 μg/ml leupeptin, and 0.5 μM PMSF. For analyses of E-cadherin *N*-glycosylation, whole cell lysates were prepared using Triton X-100/β-octyl-glucoside buffer, as described before (7). Protein concentrations were determined using the BCA protein assay (Pierce).

Plasmids—TOP Flash plasmid, which contains the wild type TCF-binding site, and FOP Flash vector, containing mutated TCF-binding sites, were purchased from Millipore. To determine functional significance of the *DPAGT1* TCF sequence, three copies of the TCF-binding sequence from the human *DPAGT1* promoter were cloned into the unique BamHI site upstream from the thymidine kinase promoter in the FOP Flash reporter plasmid using blunt end ligation.

Transient Transfection and Luciferase Assays—Plasmid DNA (2 μg), TOP Flash, FOP Flash, and FOP Flash containing 3$\times$ human DPAGT1 sequence (FOP DPAGT1) were transfected using Lipofectamine 2000 (Invitrogen) at 24 h after plating onto 35-mm plates. An empty pGL3-Basic vector was used as a control, and a reference plasmid, PSV-β-gal (0.1 μg, Promega), was used to normalize transfection efficiency. Luciferase assays were performed using a Luciferase kit according to the manufacturer's instructions (Promega). Briefly, cells were washed twice with PBS buffer and scraped with lysis reagent. The cells were centrifuged at 12,000 $\times$ g to pellet the debris. The cell extract was mixed with the luciferase assay reagent, and light emission was measured in a luminometer. The luciferase activity was assayed with duplicate samples within the linear range of the instrument. Values were normalized to β-galactosidase activity and to total protein as measured by the BCA assay using bovine serum albumin as a standard.

RNA Isolation and Real Time PCR—Total RNAs were extracted from CHO, A253, and MDCK cells using an RNeasy RNA isolation kit (Qiagen). Reverse transcriptase reactions for *DPAGT1* were performed using a SuperScript first-strand synthesis system (Invitrogen) and SuperScript III reverse transcriptase. Reactions were carried out with ABI Prism 7300 sequence detection PCR machine (Applied Biosystems) using TaqMan gene expression system, as per the manufacturer's instructions. Statistical analysis was performed using real time PCR from three independent RNA preparations, with each experiment repeated twice ($n = 6$). The p values were calculated using an unpaired t test. The cDNAs from MDCK cells were also used for detecting *ALG1* and *MGAT5* steady state mRNA levels using gene-specific TaqMan probe and primers (Applied Biosystems). 18 S was used as an endogenous control.

Deglycosylation of E-cadherin—Total cell lysates were digested with 500 units of either PNGaseF or EndoH (New England Biolabs) for 1 h at 37 °C and analyzed by Western blot. For controls, samples were incubated without enzymes.

Western Blot—Total cell lysates (1–20 μg) were fractionated on either 10 or 7.5% SDS-PAGE and blotted onto PVDF mem-

branes (Invitrogen). The membranes were blocked with 10% milk and incubated with primary antibodies in PBS/Tween (20 mM Tris, 137 mM NaCl, 0.1% Tween 20, pH 7.6) with 1% milk for 2 h at room temperature. Next, membranes were washed four times with PBS/Tween, followed by incubation with horseradish peroxidase-linked secondary antibody (1:3000). The results were visualized with ECL Plus detection reagents (Amersham Biosciences), and band analysis was performed using ImageJ (version 1.38) software.

Electrophoretic Mobility Shift Assay—Nuclear extracts were prepared according to a published procedure (36). Biotinylated oligonucleotides containing the human *DPAGT1* promoter sequences (-336 to -319 bp) were synthesized (Integrated DNA Technologies) (Fig. 1). Complementary strands without biotin were annealed to generate a double-stranded oligonucleotide probe. For the DNA mobility shift assay, the binding reaction was performed for 30 min at room temperature in 20 μl of binding buffer containing 500 fmol of biotin-labeled probe, 1 μg of poly(dI·dC)·poly(dI·dC), and nuclear extract (3–5 μg). Double-stranded oligonucleotides (sense strand 5′-CTG GGT TGC CGG GCA ACT AAC-3′ and antisense strand 5′-GTT AGT TGC CCG GCA ACC CAG-3′) at 50-fold molar excess were used as nonspecific competitors. Separation of free radiolabeled DNA from DNA-protein complexes was carried out on a 6% nondenaturing polyacrylamide gel in a standard Tris borate electrophoresis buffer at 100 V at a 4 °C. The gels were transferred to a nitrocellulose membrane at 380 mA and cross-linked with UV light for 10 min. The membranes were blocked, incubated with stabilized streptavidin, and developed with the ECL detection reagent using a light shift assay (Pierce). In the experiments where antibodies were used to characterize the protein-DNA complex, nuclear extracts were preincubated with antibodies for 20 min at room temperature before the radiolabeled probe was added, followed by 20 min of incubation with the probe.

Chromatin Immunoprecipitation (ChIP)—Chromatin in LiCl-treated A253 cells was cross-linked with 1% formaldehyde for 8 min at room temperature, sequentially washed with phosphate-buffered saline, solution I (10 mM HEPES, pH 7.5, 10 mM EDTA, 0.5 mM EGTA, 0.75% Triton X-100), and solution II (10 mM HEPES, pH 7.5, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA). Cells were incubated in lysis buffer (150 mM NaCl, 25 mM Tris, pH 7.5, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate) supplemented with protease inhibitor tablet (Roche Applied Science) and PMSF. Genomic DNA was fragmented into <500-bp pieces using a Branson 250 sonicator. Aliquots of lysates containing 200 μg of protein were used for each immunoprecipitation reaction with anti-β-catenin, anti-γ-catenin (BD Biosciences), and mouse IgG antibodies followed by adsorption to protein A/G PLUS-agarose beads (Santa Cruz Biotechnology). Precipitated DNA-protein complexes were washed sequentially with RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), high salt buffer (50 mM Tris, pH 8.0, 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), LiCl buffer (50 mM Tris, pH 8.0, 250 mM LiCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM EDTA), and TE buffer (10 mM Tris, 1 mM EDTA pH 8.0), respectively. The DNA-protein complexes were

FIGURE 1. Mammalian *DPAGT1* promoter contains TCF/LEF-binding sequences. Promoter regions from three mammalian species, human, canine, and rodent, were scanned for TCF/LEF-binding sequences. Human and hamster TCF/LEF sequences are in the forward orientation, whereas in the canine promoter sequence it is in the reverse orientation. *Numbers* indicate distances from the transcription initiation sites.

eluted with 1% SDS and 0.1 M sodium bicarbonate solution, and DNA-protein cross-link was reversed by heating the samples to 65 °C overnight. Proteins were digested with proteinase K (Sigma), and DNA was phenol/chloroform-extracted and precipitated by 100% ethanol. DNA was dissolved in 50 μl of deionized distilled water, and 10 μl was used for each real time PCR. The primers and the probe, surrounding the Wnt-responsive element, were designed using the primer express program (Applied Biosystems). They were as follows: forward primer 5′-GCTTTAGAATTTACACCATCCTTCCA-3′, reverse primer 5′-AGCCAAGGTAAACACAACTCAGTTC-3′, and TaqMan probe 6FAMATC GTA GCT TTG TTT CCGMGBNFQ.

Microscopy, Immunofluorescence, and Imaging—For indirect immunofluorescence analyses, untreated and LiCl-treated cells were fixed in 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum, and incubated with primary antibodies to either β-catenin (0.25 μg/ml) or γ-catenin (0.25 μg/ml) at room temperature for 3 h. Cells were then incubated with FITC-tagged secondary antibodies (0.1 μg/ml) and counterstained with rhodamine-phalloidin for F-actin (1:100) and with TO-PRO-3 iodide for nuclei (1:1000), all obtained from Molecular Probes. The immunostained samples were analyzed with a Zeiss confocal laser scanning microscope LSM510 META (Fluar 5×/0.25, magnification Plan-Apochromat 40×/1.3 oil differential interference contrast). For visualization of individual optical sections and for generation of Z-stacks with optical slices of 1 μm, LSM510-expert mode acquisition software was used. To ensure valid comparison of fluorescence intensities between samples, settings were fixed to the most highly stained sample and all other images were acquired at those settings.

RESULTS

DPAGT1 Promoter Contains TCF/LEF-binding Sequences—Protein *N*-glycosylation has been shown to be regulated with cell proliferation and differentiation (8, 14, 37). Thus, we reasoned that as a key regulator of *N*-glycosylation, the *DPAGT1* gene is likely a downstream target of signaling pathways with roles in development. Because Wnt/β-catenin signaling plays pivotal roles in many developmental processes, we analyzed the *DPAGT1* promoter regions from three mammalian species, human, canine, and hamster, for the presence of TCF/LEF-binding sites. All three *DPAGT1* promoters contained the TCF/LEF consensus sequences (Fig. 1). In human and hamster, the TCF/LEF-binding sites were in a forward orientation, although in canine, the consensus sequence was in a reverse orientation. Because TCF/LEF sequences function in both orientations, the presence of the TCF/LEF-binding sites in
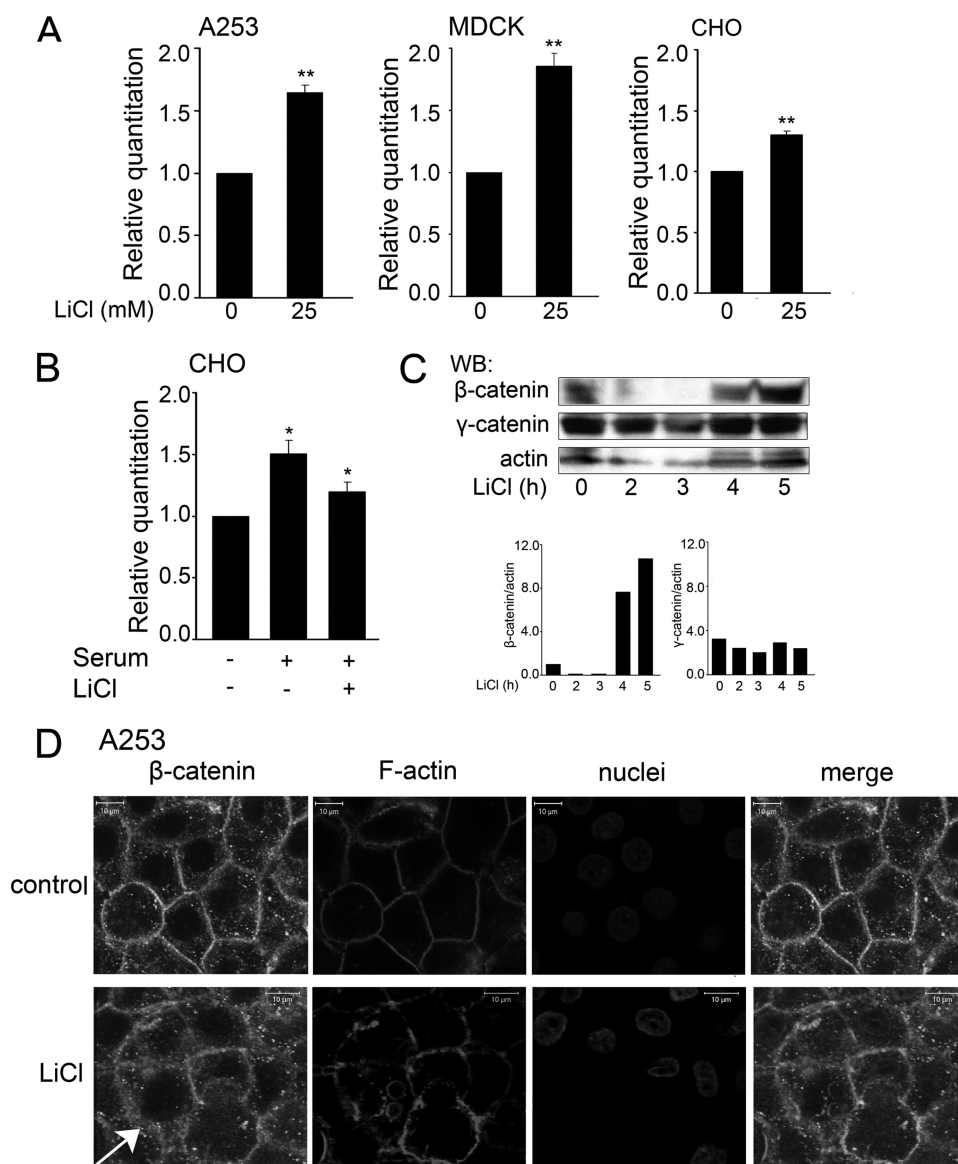
FIGURE 2. **Lithium chloride up-regulates *DPAGT1* transcript levels.** *A,* total RNAs were prepared from A253, MDCK, and CHO cells in the absence or presence of 25 mM LiCl and used for cDNA synthesis to assess *DPAGT1* expression by real time RT-PCR. Gene expression profiles were generated by normalizing *DPAGT1* expression with the 18 S ribosomal RNA gene and comparing it with the gene expression of untreated cells. Statistical analysis was performed using real time PCR from two independent RNA preparations, with each experiment being repeated twice (*n* = 4) (**, *p* < 0.01). *B,* serum counteracts the effects of LiCl on *DPAGT1* expression. Total RNA lysates were prepared from CHO cells that were serum-starved for 24 h, followed by serum stimulation in the absence or presence of 25 mM LiCl. RNA lysates were used for cDNA synthesis to assess *DPAGT1* expression by real time RT-PCR. Gene expression profiles were generated by normalizing *DPAGT1* expression to housekeeping gene *18 S* and comparing it with the gene expression of untreated cells. Statistical analysis was performed using RT-PCR from three independent RNA preparations, with each experiment being repeated twice (*n* = 6) (*, *p* < 0.05). *C,* Western blot (*WB*) analysis of β-catenin and γ-catenin expression following treatment of A253 cells with LiCl. *Bar graph,* fold changes in β-catenin and γ-catenin levels after addition of LiCl were determined by normalizing to actin in two different experiments. *D,* treatment of cells with LiCl results in an increased cytoplasmic localization of β-catenin. Untreated and LiCl-treated cells were grown in chamber slides to confluence and processed for indirect immunofluorescence staining using an antibody to β-catenin. Cells were counterstained for F-actin with rhodamine-phalloidin and for nuclei with TO-PRO-3 iodide. In control cells, β-catenin displayed more cell-cell border staining compared with LiCl-treated cells, where it was more cytoplasmic (*arrow*). Shown are 1-μm confocal *x-y* sections. *Size bars,* 10 μm. These data are representative of two different experiments.

mammalian *DPAGT1* promoters suggested that this gene was a target of the Wnt/β-catenin signaling pathway. In contrast, another gene in the *N*-glycosylation pathway, *ALG1*, that functions in the addition of the first mannose residue to the dolichol-pyrophosphate-GlcNAc2 intermediate in the

endoplasmic reticulum did not contain TCF-binding sequences.

DPAGT1 Transcript Levels Are Regulated by Activators and Inhibitors of Wnt Signaling—The Wnt/β-catenin signaling pathway is regulated by different input signals that serve as both its activators and inhibitors (16). Among the known activators of the canonical Wnt pathway is LiCl, which functions as an inhibitor of the GSK3β activity (38). When in complex with adenomatous polyposis coli and axin, GSK3β phosphorylates serine/threonine (Ser/Thr) residues in the N-terminal region of β-catenin, rendering it susceptible for degradation through the ubiquitin pathway. Upon inhibition of GSK3β, β-catenin accumulates in the cytoplasm and translocates to the nucleus where it interacts with TCF and activates target genes (22). To determine whether LiCl affected *DPAGT1* transcript levels, we treated A253 cells with 5 and 25 mM LiCl and found a dose-dependent increase in *DPAGT1* expression (data not shown). Because 25 mM LiCl had a more pronounced effect, we used this concentration in all subsequent studies. Treatment of A253, MDCK, and CHO cells with 25 mM LiCl resulted in the up-regulation of *DPAGT1* transcript levels, although to different extents (Fig. 2*A*). The most pronounced induction was detected in MDCK cells (85%), followed by A253 cells (65%) and CHO cells (30%). These cell type variations in the LiCl induction of *DPAGT1* transcription are likely to reflect cell type differences. Because LiCl has been shown to antagonize the effects of serum on the expression of early growth response genes, we examined *DPAGT1* transcript levels in CHO cells treated with serum in the absence or presence of LiCl. Indeed, although serum alone up-regulated *DPAGT1* transcripts by 50%, this effect was abrogated by LiCl (Fig. 2*B*). As expected, induction of *DPAGT1* transcript levels in LiCl-treated A253 cells correlated with a 10-fold increase in β-catenin abundance within 5 h of LiCl addition (Fig. 2*C*). In contrast to β-catenin, 25 mM LiCl did not significantly affect the cellular levels of γ-catenin through-

out the time course of the experiment (Fig. 2*C*). Immunofluorescence localization of β-catenin in A253 cells revealed that in addition to being present at cell-cell interfaces, it was also found in the cytoplasm. The cytosolic fraction of β-catenin was augmented in cells treated with LiCl, supporting its role as an activator of Wnt/β-catenin signaling (Fig. 2*D*, *LiCl*, *arrow*).

To confirm that the effects of LiCl on *DPAGT1* expression were a consequence of the activation of the canonical Wnt pathway, we used one of the receptor ligands of Frizzled, Wnt3a, to activate Wnt signaling. Treatment of A253 cells with 3 μg of Wnt3a resulted in a 60% increase in the *DPAGT1* transcript levels, suggesting that the Wnt pathway was an upstream activator of this gene (Fig. 3*A*). In CHO cells, Wnt3a caused a modest (22%) induction of *DPAGT1* transcript abundance (Fig. 3*A*). In contrast, addition of Wnt3a had no discernable effect on the *DPAGT1* transcript levels in MDCK cells. Because Wnt3a was added to cells at a single time point, the observed lack of response in MDCK cells was likely to reflect experimental conditions. MDCK cells have been shown to be sensitive to Wnt3a in co-cultures with NIH-3T3 cells expressing Wnts (39). Therefore, we tested whether growth of MDCK cells in the presence of 50% conditioned medium isolated from either L-mouse fibroblasts or L-mouse fibroblasts stably transfected with Wnt3a would lead to the induction of the *DPAGT1* transcript. As shown in Fig. 3*B*, following growth for 24 h with 50% conditioned medium containing Wnt3a, *DPAGT1* transcript levels increased by 70%. For a negative control, we used the *N*-glycosylation gene, *ALG1*, which functions downstream from *DPAGT1* in the dolichol pathway in the endoplasmic reticulum and encodes the first mannosyltransferase and which lacks the TCF/LEF-binding sequence in its promoter. As expected, Wnt3a did not induce the *ALG1* transcript (Fig. 3*B*). We also examined transcript levels of another *N*-glycosylation gene, *MGAT5*, which encodes *N*-acetylglucosaminyltransferase V (Mgat5 or GnT-V) that functions in the addition of branched *N*-glycans in the Golgi. The *MGAT5* promoter has four sequences resembling TCF/LEF, although with different degrees of fidelity. They include a sequence beginning at −192 bp from the transcription start site with two mismatches, a site at −410 with one mismatch, a site at −1312 bp with one mismatch, and an exact TCF/LEF sequence at −1596 bp. Under the conditions of our study, however, Wnt3a did not induce *MGAT5* transcript levels (Fig. 3*B*).

In A253 cells, the Wnt3a-dependent increase in the *DPAGT1* transcript levels was abrogated by the Wnt inhibitor, Dkk-1 (Fig. 3*C*). Treatment of A253 cells with Dkk-1 alone caused a 24% down-regulation of *DPAGT1* transcripts, suggesting the existence of endogenous Wnt activity in these cells (Fig. 3*C*). Likewise, MDCK cells were sensitive to Dkk-1 treatment, which reduced *DPAGT1* transcripts by 56% (Fig. 3*D*).

Similar to LiCl-treated A253 cells, incubation of MDCK cells with 50% conditioned medium containing Wnt3a was associated with an increased abundance of β-catenin (Fig. 3*E*, β-*cat*). The steady-state levels of γ-catenin were also augmented in Wnt3a-treated cells, although to a much lesser degree than β-catenin (Fig. 3*E*, γ-*cat*).
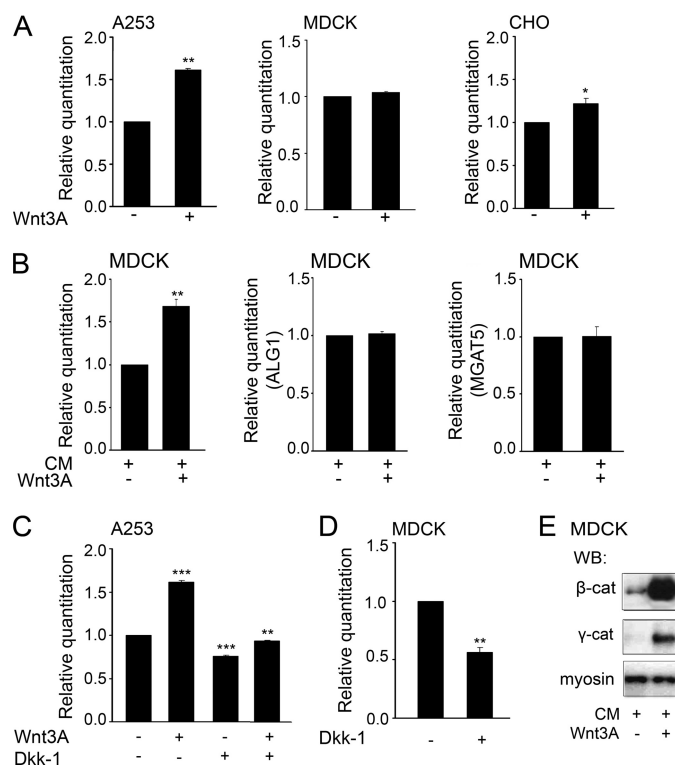

FIGURE 3. **Transcriptional expression of *DPAGT1* is stimulated by Wnt3a and abrogated by Dkk-1.** *A*, total RNAs isolated from A253, MDCK, and CHO cells treated with Wnt3a were used for cDNA synthesis to assess *DPAGT1* expression by real time RT-PCR. Gene expression profiles were generated by normalizing *DPAGT1* expression with housekeeping gene *18 S* and comparing it with the gene expression of untreated cells. Statistical analysis was performed using real time PCR from three independent RNA preparations, with each experiment being repeated twice ($n = 6$) (*, $p < 0.05$; **, $p < 0.01$). Results represent one of three independent experiments. *B*, total RNAs were isolated from MDCK cells grown in CM in the presence or absence of Wnt3a. RNAs were used for cDNA synthesis to assess *DPAGT1*, *ALG1*, and *MGAT5* expression by real time RT-PCR. Gene expression profiles were generated by normalizing *DPAGT1*, *ALG1*, and *MGAT5* expression with housekeeping gene *18 S* and comparing it with the gene expression of untreated cells. Statistical analysis was performed using real time PCR from three independent RNA preparations, with each experiment being repeated twice ($n = 6$) (**, $p < 0.01$). Results represent one of three independent experiments. *C*, total RNA lysates were prepared from A253 cells treated with either with Wnt3a, Dkk-1, or both and used for cDNA synthesis to assess *DPAGT1* expression by RT-PCR. Gene expression profiles were generated by normalizing *DPAGT1* expression with housekeeping gene *18 S* and comparing it with the gene expression of untreated cells. Statistical analysis was performed using RT-PCR from three independent RNA preparations, with each experiment being repeated twice ($n = 6$) (**, $p < 0.01$; ***, $p < 0.001$). *D*, total RNAs were isolated from MDCK cells treated with Dkk-1 and used for cDNA synthesis to assess *DPAGT1* expression by RT-PCR. Gene expression profiles were generated by normalizing *DPAGT1* expression with housekeeping gene *18 S* and comparing it with the gene expression of untreated cells. Statistical analysis was performed using real time PCR from three independent RNA preparations, with each experiment being repeated twice ($n = 6$) (**, $p < 0.01$). *E*, equal amounts of total cell lysate protein from MDCK cells, grown in CM in the presence or absence of Wnt3a, were analyzed for β-catenin and γ-catenin expression by Western blot (*WB*). Myosin served as a loading control.

TCF/LEF-binding Sites in the DPAGT1 Promoter Interact with Tcf and β-Catenin in Vitro and in Vivo—To validate the effect of Wnt activation on *DPAGT1* expression, we examined the DPAGT1 TCF/LEF sequence for the ability to bind the Tcf-β-catenin protein complex. When a biotinylated human *DPAGT1* promoter sequence spanning the TCF-binding sites was tested for interaction with nuclear extracts from A253 cells, a robust protein-DNA complex was detected (Fig. 4*A*). The
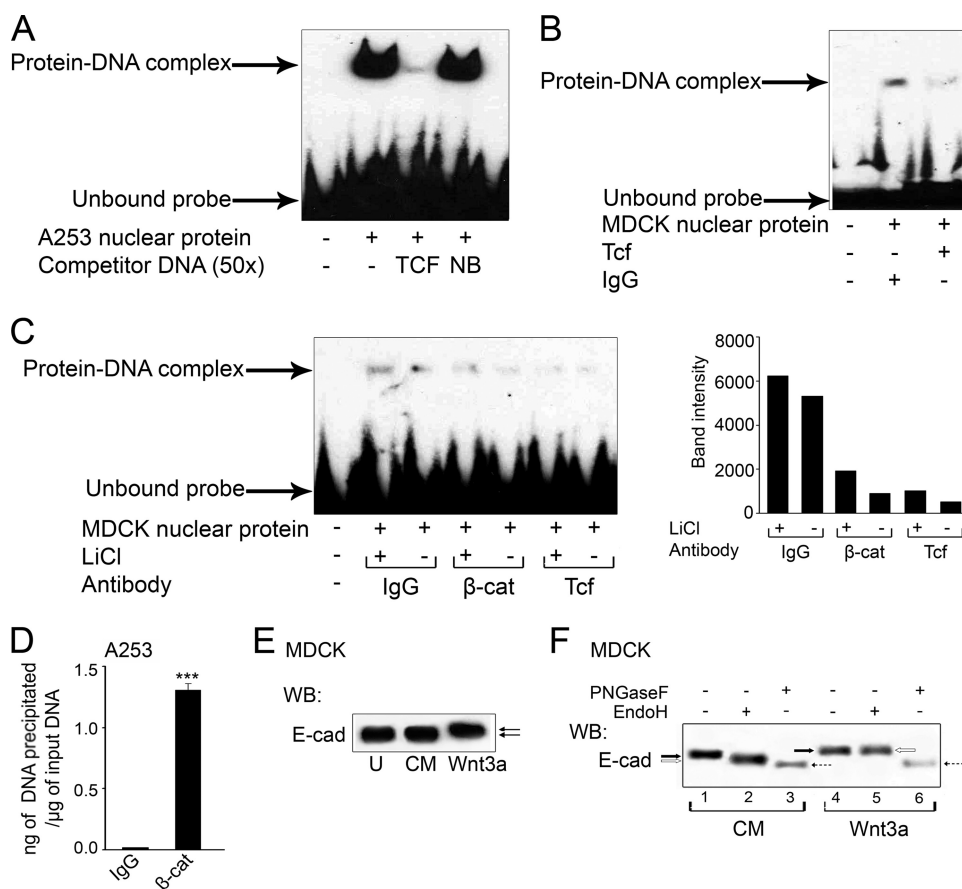
FIGURE 4. TCF/LEF-binding sequence in the *DPAGT1* promoter interacts with TCF and β-catenin *in vitro* and *in vivo*. *A*, DNA shift assay was performed using the nuclear extract (5 μg) from A253 cells that was incubated with a biotinylated (−336 to −319) sequence from the human *DPAGT1* promoter. Where indicated, competitor TCF or unrelated probes (*NB*) were used (*3rd* and *4th lanes*). Data shown represent one of four independent experiments. *B*, DNA mobility shift assay was carried out with a nuclear protein extract (5 μg) from MDCK cells and a canine-biotinylated (−244 to −221) *DPAGT1* promoter sequence in the presence of either IgG (1 μg) or Tcf antibody (1 μg). Results are one of three independent experiments. *C*, gel shift assay was performed with a nuclear protein extract (5 μg) from MDCK cells, either untreated or treated with LiCl, and a canine biotinylated *DPAGT1* promoter sequence in the presence of either IgG (1 μg), β-catenin (*β-cat*) antibody (1 μg), or TCF antibody (1 μg). These results are one of two independent experiments. *Bar graph*, band intensities were calculated in comparison to the IgG control in the presence of LiCl. The data are representative of three independent experiments. *D*, ChIP assay was performed on chromatin samples from A253 cells using either an antibody against β-catenin or IgG (negative control). Real time PCRs were carried out using primers and the TaqMan probe specific for the promoter region of *DPAGT1*. An unrelated sequence was used as a negative control. *E*, equal amounts of total cell lysate protein from untreated MDCK cells (*U*), and cells grown in CM in the presence or absence of Wnt3a, were analyzed for E-cadherin expression by WB. *F*, total cell lysates from cells, grown in CM in the presence or absence of Wnt3a, were treated with EndoH and PNGaseF and analyzed by Western blot (*WB*). The results depict one of three independent experiments. *E-cad*, E-cadherin.

Tcf, either in the presence or absence of LiCl (Fig. 4*C*, *IgG versus β-cat versus Tcf*). As expected, LiCl did not affect the abundance of the protein-DNA complex in the competition study with the Tcf antibody (Fig. 4*C*, *Tcf*). Because both Tcf and β-catenin were able to compete off the nuclear complex at the TCF/LEF-binding site, this indicated that β-catenin interacted with Tcf at this site in the *DPAGT1* promoter *in vitro*.

To determine whether the Tcf-β-catenin complex was also present at the *DPAGT1* promoter *in vivo*, we carried out ChIP assays with chromatin isolated from A253 cells treated with LiCl and an antibody to β-catenin. Relative to the IgG control, there was a 60-fold increase in the amount of β-catenin bound to the *DPAGT1* TCF/LEF sequence (Fig. 4*D*). We conclude that the TCF/LEF-binding site in the *DPAGT1* promoter interacts with Tcf and β-catenin *in vitro* and *in vivo*.

Up-regulation of DPAGT1 by Wnt3a Leads to Altered N-Glycosylation of E-cadherin—Our previous studies have shown that *DPAGT1* impacts E-cadherin *N*-glycosylation, both quantitatively and qualitatively (10, 15). High levels of *DPAGT1* expression correlate with extensive *N*-glycosylation of E-cadherin with complex *N*-glycans, whereas down-regulation of *DPAGT1* leads to reduced *N*-glycosylation status of E-cadherin concomitant with the modification with high mannose/hybrid *N*-glycans.

To align the Wnt-dependent transcriptional activation of *DPAGT1* with *N*-glycosylation of E-cadherin, we examined sensitivity of E-cadherin to PNGaseF and EndoH following the induction of *DPAGT1* with Wnt3a. PNGaseF is an amidase that removes most *N*-glycans from *N*-glycoproteins, although EndoH is an endoglycosidase that removes high mannose/hybrid *N*-glycans. Western blot analyses showed that E-cadherin from MDCK cells grown in the presence of 50% conditioned medium (CM) containing Wnt3a for 72 h migrated with a higher molecular size compared with E-cadherin from control cells, grown in the presence of 50% conditioned medium without Wnt3a or from untreated cells (Fig. 4*E*). PNGaseF treatment of E-cadherin from Wnt3a cells resulted in mobility shift that reduced its molecular size to that of PNGaseF-treated E-cadherin from CM cells (Fig. 4*F*, *lanes 3* and *6, dashed*

complex was competed off by the TCF sequence but not by an unrelated sequence from the *DPAGT1* promoter (Fig. 4*A*). Similar results were obtained with nuclear extracts from MDCK and CHO cells (data not shown). To determine whether Tcf and β-catenin proteins were present in complex with the TCF binding region of the *DPAGT1* promoter, we carried out competition studies with antibodies to Tcf and β-catenin. Incubation of nuclear extracts from MDCK cells with the Tcf-specific antibody greatly diminished the abundance of the protein-DNA complex compared with the nuclear extracts incubated in the presence of IgG alone (Fig. 4*B*). Furthermore, the levels of protein-DNA complexes were greater when nuclear extracts were isolated from MDCK cells treated with LiCl compared with untreated cells (Fig. 4*C*, *IgG*). The abundance of protein-DNA complexes was diminished by competition with β-catenin and
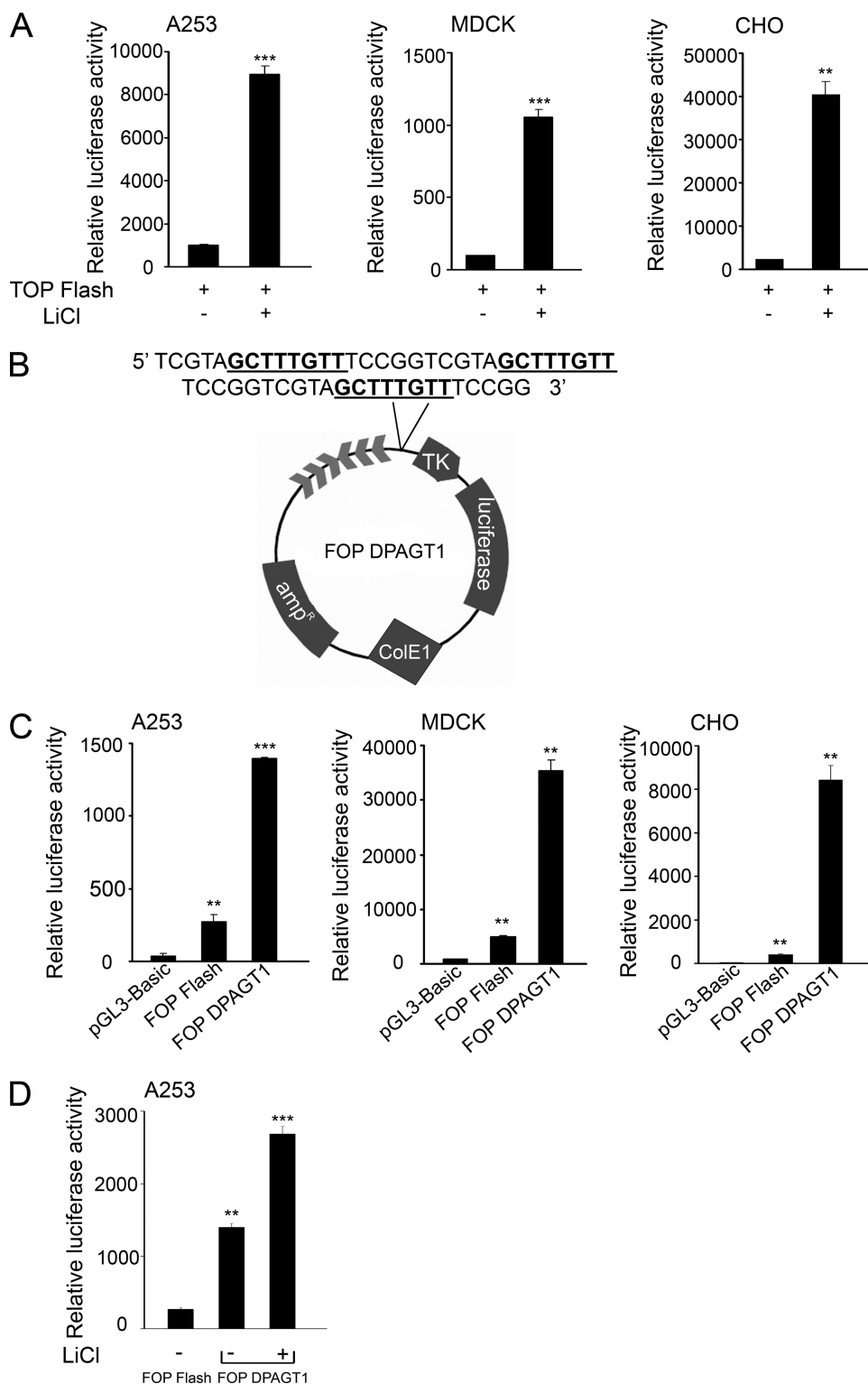
FIGURE 5. **TCF/LEF-binding sequence from the *DPAGT1* promoter drives luciferase reporter gene expression.** *A*, luciferase reporter gene under the regulation of three tandem copies of TCF/LEF-responsive elements in the TOP Flash vector was transfected into either A253, MDCK, or CHO cells, and cells were grown for 24 h in the absence or presence of LiCl, and extracts were analyzed for luciferase activity after 48 h. *Error bars* indicate S.E. (**, $p < 0.01$; ***, $p < 0.001$). *B*, schematic of FOP DPAGT1, the FOP Flash vector with three copies of the TCF/LEF-responsive element from the *DPAGT1* promoter upstream of the luciferase reporter gene. *C*, either FOP DPAGT1, FOP Flash, or pGL3-Basic were transfected into A253, MDCK, or CHO cells, and cells were grown for 48 h. Extracts were analyzed for luciferase activity. *Error bars* indicate S.E. (**, $p < 0.01$; ***, $p < 0.001$). *D*, FOP DPAGT1 was transfected into A253 cells, and cells were grown in the presence of LiCl for 24 h. FOP Flash was used as a negative control. Transfections were normalized using the Psv-β-galactosidase control vector. Extracts were analyzed for luciferase activity after 48 h. The values depicted are S.E. of three independent experiments performed in duplicate ($n = 6$) (**, $p < 0.01$; ***, $p < 0.001$).

arrows). Moreover, E-cadherin from control cells was sensitive to EndoH, indicating that, in addition to complex *N*-glycans, it had high mannose/hybrid *N*-glycans (Fig. 4*F*, *lanes 1* and *2, arrows*). In contrast, E-cadherin from Wnt3a-treated cells exhibited little sensitivity to EndoH (Fig. 4*F*, *lanes 4* and *5, arrows*), resembling the scenario of E-cadherin from proliferating MDCK cells and A253 cancer cells, where it is modified primarily with PNGaseF-sensitive, complex *N*-glycans (7, 10). We conclude that up-regulation of *DPAGT1* in response to Wnt3a treatment results in increased *N*-glycosylation of E-cadherin with complex *N*-glycans.

TCF Sequence from the DPAGT1 Promoter Drives Expression of the Luciferase Reporter Gene—The results thus far strongly suggested that *DPAGT1* expression was regulated in response to Wnt/β-catenin signaling through the binding of Tcf/β-catenin to the TCF/LEF sequence in its promoter. To validate that the DPAGT1 TCF/LEF promoter sequence was biologically functional, we examined whether it could influence expression of a luciferase reporter. As expected, transfection of CHO, MDCK, and A253 cells with a luciferase reporter vector containing three wild type TCF/LEF-binding sites (TOP Flash) caused a robust increase (10–20-fold) in the luciferase activity upon Wnt stimulation with LiCl (Fig. 5*A*). We next transfected these cells with either a vector that had three mutant TCF/LEF-binding sites (FOP Flash) or with a FOP Flash vector bearing three TCF/LEF-binding sites from the *DPAGT1* promoter (FOP DPAGT1) upstream of the thymidine kinase promoter sequence (Fig. 5*B*). All three cell lines transfected with the FOP DPAGT1 vector showed significantly augmented luciferase activity compared with cells transfected with either FOP Flash alone or a negative control plasmid, pGL3-Basic, lacking TCF-binding sites (Fig. 5*C*). The variations in the luciferase activities among the cell
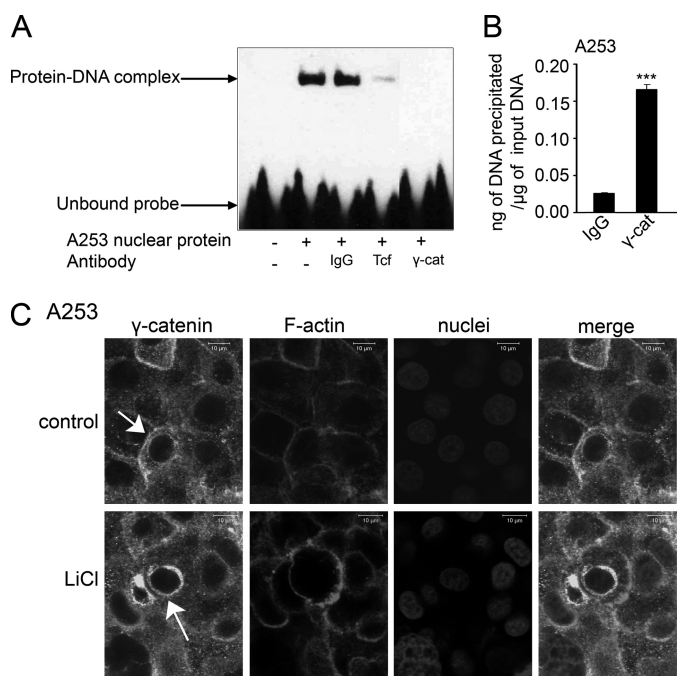
A

Protein-DNA complex →

Unbound probe →

| A253 nuclear protein | – | + | + | + | + |
| Antibody | – | – | IgG | Tcf | γ-cat |

B

A253

ng of DNA precipitated /μg of input DNA

0.20
0.15
0.10
0.05
0.00

IgG   γ-cat   ***

C   A253

	γ-catenin	F-actin	nuclei	merge
control				
LiCl				

FIGURE 6. ***DPAGT1 promoter binds γ-catenin *in vitro* and *in vivo*.*** *A,* DNA mobility shift assay was carried out with a nuclear protein extract (5 μg) from A253 cells and a human *DPAGT1* promoter sequence (−336 to −319) in the presence of either IgG (1 μg), Tcf antibody (1 μg), or γ-catenin (γ-*cat*) antibody (1 μg). Results are one of three independent experiments. *B,* ChIP analysis using an antibody against γ-catenin was performed on chromatin samples from A253 cells. Real time PCR was carried out using primers and the TaqMan probe specific for the promoter region of *DPAGT1.* Genomic DNA precipitated by IgG was used as a negative control. Statistical analysis was performed using real time PCR from two independent RNA preparations, with each experiment being repeated twice ($n = 4$) (**, $p < 0.01$; ***, $p < 0.001$). *C,* untreated and LiCl-treated cells were grown in chamber slides to confluence and processed for indirect immunofluorescence staining using an antibody to γ-catenin. Cells were counterstained for F-actin with rhodamine-phalloidin and for nuclei with TO-PRO-3 iodide. Shown are 1 μm confocal *x-y* sections. *Size bars,* 10 μm. These data are one of two independent experiments.

lines most likely reflected the differences in cell context. Moreover, A253 cells transfected with FOP DPAGT1 displayed a further increase in the luciferase activity following stimulation with LiCl (Fig. 5*D*). These results confirmed that the Wnt/β-catenin pathway was a direct transcriptional activator of the *DPAGT1* gene.

TCF/LEF-binding Site in the DPAGT1 Promoter Interacts with γ-Catenin—Another protein with the Tcf/Lef binding domain highly homologous to β-catenin is γ-catenin/plakoglobin. Although γ-catenin has been shown to interact with Tcf/Lef, its binding pattern differs from that of β-catenin (40). Thus, we sought to determine whether γ-catenin could also interact with the *DPAGT1* TCF/LEF binding region. Antibodies to γ-catenin were effective in competing off the protein-DNA complex at the *DPAGT1* TCF/LEF-binding site compared with IgG (Fig. 6*A*), indicating that γ-catenin was able to interact with this site *in vitro*. To assess if binding of γ-catenin to the *DPAGT1* promoter occurred *in vivo*, we performed a ChIP assay with chromatin isolated from A253 cells treated with LiCl and an antibody to γ-catenin. As shown in Fig. 6*B*, there was a 7-fold increase in the amount of γ-catenin bound to the *DPAGT1* TCF/LEF sequence compared with the IgG control. However, in contrast to β-catenin, this binding of γ-catenin to

the *DPAGT1* promoter was not inducible by LiCl (Fig. 2*C*). On the other hand, there was a detectable increase in the abundance of γ-catenin after stimulation with Wnt3a (Fig. 3*E*), confirming its role in Wnt signaling (41). Immunofluorescence staining of γ-catenin in A253 cells revealed that its cytoplasmic localization was not impacted by treatment with LiCl (Fig. 6*C*). Nonetheless, in A253 cells, γ-catenin displayed more pronounced cytoplasmic localization than β-catenin (Fig. 2). Interestingly, a significant fraction of γ-catenin was detected in a perinuclear region (Fig. 6*C, arrow*). These results suggest that γ-catenin interacts with the *DPAGT1* promoter through a Wnt-dependent mechanism distinct from that of β-catenin.

DISCUSSION

Our studies reveal that the first *N*-glycosylation gene, *DPAGT1*, is a target of the Wnt/β-catenin signaling pathway. Under conditions of Wnt activation, *DPAGT1* transcription is up-regulated though the binding of β-catenin to Tcf at the TCF/LEF-binding site in the *DPAGT1* promoter. Because Wnt signaling plays pivotal roles in cell proliferation and differentiation, and because *DPAGT1* and the *N*-glycosylation status of proteins are regulated with growth and differentiation, our findings provide, at least in part, a molecular explanation for these observations.

Treatment of nonmalignant CHO and MDCK cells, as well as A253 cancer cells, with a β-catenin stabilizer, LiCl, resulted in an up-regulation of *DPAGT1* transcript levels concomitant with an increase in the cytoplasmic pool of β-catenin (Fig. 2). Similar up-regulation of *DPAGT1* transcription was obtained upon the addition of a Frizzled ligand, Wnt3a, and this effect was abrogated by the Wnt inhibitor, Dkk-1 (Fig. 3).

DNA binding assays using the TCF/LEF binding region from the *DPAGT1* promoter validated this finding by showing the presence of both Tcf and β-catenin *in vitro* (Fig. 4). Likewise, the ChIP assay confirmed the presence of β-catenin at the *DPAGT1* promoter *in vivo* (Fig. 4*D*). Finally, the TCF/LEF region from the *DPAGT1* promoter was shown to drive the expression of the luciferase reporter gene in all three cell lines (Fig. 5). Collectively, our studies provide the first evidence that *DPAGT1* is regulated by Wnt/β-catenin signaling.

We note that another key *N*-glycosylation gene, *MGAT5*, which functions in the Golgi in the addition of branched *N*-glycans (1) and which has been recognized to promote cancer metastasis (8, 9, 43, 44), is not a target of the Wnt/β-catenin pathway (Fig. 3*B*).

Interestingly, LiCl abrogated the up-regulation of *DPAGT1* expression by serum (Fig. 2*B*). To date, the antagonistic effect of Wnt activation on serum response has been reported for early growth response genes (45). Although *DPAGT1* has been shown to behave like an early growth response gene in yeast, in mammalian systems it does not respond to mitogenic stimulation in a manner characteristic of this class of genes.[3] Thus, our findings extend the antagonistic effects of Wnt activation on mitogenic stimulation to a broader category of genes.

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[3] P. K. Sengupta and M. A. Kukuruzinska, unpublished observations.

Regulation of N-Glycosylation by Wnt Signaling

The Wnt/β-catenin pathway has been increasingly recognized to play critical roles in cellular metabolism (46). Specifically, Wnt signaling has been implicated in a number of distinct metabolic processes, including amino acid, lipid, and glucose sensing (47). Through its effects on expression of key metabolic proteins, genes, and transcription factors, Wnt signaling is thought to coordinate the metabolic flux under normal physiological conditions. In some cases, Wnt signaling regulates metabolism via its effects on a post-translational level. For instance, Wnt regulates a cluster of glycolytic enzymes, comprised of aldolase, phosphofructokinase, and hexokinase via the GSK-Axin destruction complex. In macrophages, activation of the Wnt/β-catenin pathway has been shown to depend on glucose availability and the hexose biosynthetic pathway. Moreover, N-glycosylation itself plays important roles in glucose sensing, as the initial substrate for the *DPAGT1* gene product, N-acetylglucosamine-1-phosphate transferase, is UDP-GlcNAc, a product to the hexosamine pathway (47).

Although our studies show that Wnt/β-catenin signaling impacts N-glycosylation via *DPAGT1*, N-glycosylation itself has been shown to affect Wnt signaling via modification of Wnt ligands (35). Because *DPAGT1* determines the N-glycosylation status of proteins (12, 13), and because Wnts require N-glycosylation for activity (35), the Wnt/β-catenin pathway is likely to be *DPAGT1*-dependent.

An additional interaction between Wnt/β-catenin signaling and N-glycosylation occurs at the level of E-cadherin-mediated cell-cell adhesion (48). *DPAGT1* expression controls the N-glycosylation status of E-cadherin, which, in turn, impacts the molecular organization of AJs, tight junctions, and their cytoskeletal interactions (7, 15, 49). This study shows that the Wnt/β-catenin-dependent up-regulation of *DPAGT1* transcription leads to altered N-glycosylation of E-cadherin, producing a shift from a mixture of high mannose/hybrid and complex N-glycans to almost exclusively complex structures (Fig. 4, E and F). This suggests that in addition to controlling the extent of protein N-glycosylation, *DPAGT1* affects the types of N-glycans. We and others have shown that extensive N-glycosylation of E-cadherin with complex N-glycans inhibits cell-cell adhesion (7, 50). Thus, our present findings suggest that *DPAGT1* coordinates pro-proliferative effects of Wnt/β-catenin signaling with weak cell-cell adhesion. The schematic depicting how DPAGT1 integrates Wnt signaling with E-cadherin adhesion is shown in Fig. 7. Furthermore, E-cadherin itself is a downstream target of Wnt/β-catenin signaling, which represses its expression on a transcriptional level via Twist, Snail, and Slug (6, 51, 52). Thus, activation of Wnt/β-catenin signaling suppresses intercellular adhesion on two levels as follows: through up-regulation of *DPAGT1,* leading to extensive N-glycosylation of E-cadherin, and through transcriptional inhibition of E-cadherin. In addition, functional AJs require interaction between E-cadherin and β-catenin, and once dissociated from AJs, β-catenin can feed into the Wnt pathway. Such a scenario may be the underlying cause of many epithelial cancers (27, 42).

Our present studies also suggest that γ-catenin is a potential transcriptional activator of *DPAGT1* through a mechanism that is different from that of β-catenin (Fig. 6). Although both
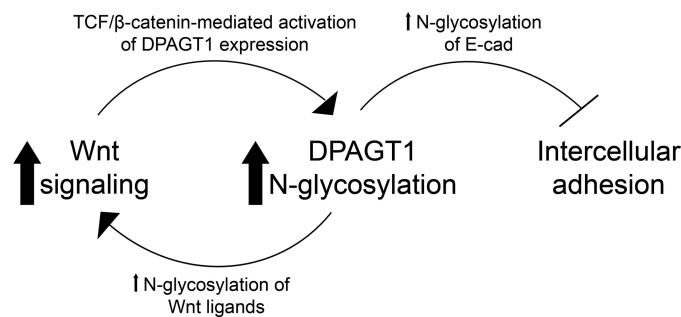

FIGURE 7. **Schematic illustration of how *DPAGT1* coordinates Wnt signaling with E-cadherin adhesion.** In response to Wnt activation, β-catenin is stabilized and translocates to the nucleus where it binds to Tcf at the *DPAGT1* promoter and activates *DPAGT1* expression. Increased expression of *DPAGT1* promotes N-glycosylation of Wnt ligands and Wnt activity, which further up-regulates *DPAGT1* expression. A Wnt-dependent increase in *DPAGT1* expression leads to an increased N-glycosylation of E-cadherin (*E-cad*) with complex N-glycans, which interferes with intercellular adhesion.

β-catenin and γ-catenin interact with E-cadherin in a mutually exclusive manner, their association with hypoglycosylated E-cadherin drives the interaction of AJs with distinct cytoskeletal components and with different functional outcomes (49). We have shown before that the *DPAGT1*-dependent remodeling of AJs involves changes in the association of γ-catenin with E-cadherin scaffolds, with mature hypoglycosylated AJs recruiting more γ-catenin where it acts as a junctional stabilizer (7, 10, 15). The abundance of γ-catenin is prominently diminished in immature, highly N-glycosylated AJs from a subset of oral squamous cell carcinoma tissues overexpressing *DPAGT1* (10). In light of our present results with A253 cells, which also overexpress *DPAGT1* and form weak AJs, it is possible that in cancer tissues, γ-catenin is not sequestered by immature AJs, which allows it to accumulate in the cytoplasm and to translocate to the nucleus where it up-regulates *DPAGT1*. Interestingly, in A253 cells, γ-catenin appeared to accumulate in the perinuclear region, suggesting that its entry to the nucleus may be a regulated, multistep process (Fig. 6C). Collectively, our studies provide evidence that both β-catenin and γ-catenin may serve as links coordinating N-glycosylation with Wnt and intercellular adhesion, although in different cellular contexts.

In addition to E-cadherin, the Wnt/β-catenin pathway interacts with a number of other signaling networks, including Notch, Hedgehog, transforming growth factor β (TGFβ), and bone morphogenetic protein signaling (16). To date, at least 80 target genes of Wnt/β-catenin signaling have been identified. Some of the prominent transcriptional targets include the genes that encode cyclin D1, c-Myc, c-Jun, mitochondrial cytochrome oxidase II, E-cadherin, fibronectin, and matrix metalloproteinases (46). Notably absent among these transcriptional targets are the genes involved in cellular metabolism. Our studies now add *DPAGT1*, a key gene in the metabolic pathway of protein N-glycosylation, to the list of target genes of the canonical Wnt/β-catenin pathway.

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