PAR1b Promotes Cell–Cell Adhesion and Inhibits Dishevelled-mediated Transformation of Madin-Darby Canine Kidney Cells[□]

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Mammalian Par1 is a family of serine/threonine kinases comprised of four homologous isoforms that have been associated with tumor suppression and differentiation of epithelial and neuronal cells, yet little is known about their cellular functions. In polarizing kidney epithelial (Madin-Darby canine kidney [MDCK]) cells, the Par1 isoform Par1b/MARK2/EMK1 promotes the E-cadherin-dependent compaction, columnarization, and cytoskeletal organization characteristic of differentiated columnar epithelia. Here, we identify two functions of Par1b that likely contribute to its role as a tumor suppressor in epithelial cells. 1) The kinase promotes cell–cell adhesion and resistance of E-cadherin to extraction by nonionic detergents, a measure for the association of the E-cadherin cytoplasmic domain with the actin cytoskeleton, which is critical for E-cadherin function. 2) Par1b attenuates the effect of Dishevelled (Dvl) expression, an inducer of wnt signaling that causes transformation of epithelial cells. Although Dvl is a known Par1 substrate *in vitro*, we determined, after mapping the PAR1b-phosphorylation sites in Dvl, that PAR1b did not antagonize Dvl signaling by phosphorylating the wnt-signaling molecule. Instead, our data suggest that both proteins function antagonistically to regulate the assembly of functional E-cadherin-dependent adhesion complexes.

INTRODUCTION

Mammalian proteins known by the synonyms Par1/MARK/EMK/kp78 comprise a family of four homologous serine/threonine kinases. Par1 was first described as tumor suppressor by Parsa (1988) who in 1988 characterized a 78-kDa protein identical to Par1a as a cell surface antigen of normal pancreatic epithelia that was absent in pancreatic cancers. Recently, Beghini *et al.* (2003) reported altered MARK4 expression to be associated with gliomas. Despite this evidence for an involvement of Par1 kinases in carcinogenesis, no cellular functions have been identified that could explain a role of Par1 in tumor suppression.

Evidence from work in *Drosophila* and cultured epithelial cells indicate that Par1 is essential for establishing and maintaining columnar epithelial monolayers. Par1 knockdown resulted in a disorganized, often multilayered follicle epithelium in *Drosophila*, although loss of apico-basolateral polarity in individual cells was infrequent (Cox *et al.*, 2001; Doerflinger *et al.*, 2003). Similarly, when the mammalian Par1 isoform Par1b/EMK1/MARK2 was depleted from polarizing kidney epithelial cells (Madin-Darby canine kidney [MDCK]), the developing epithelial monolayer exhibited apico-basolateral polarity but seemed disorganized, and cells failed to compact and to acquire a columnar cell shape

(Bohm *et al.*, 1997; Cohen *et al.*, 2004; Suzuki *et al.*, 2004). Par1 not only promotes the maintenance of epithelial monolayers but also the organized remodeling of cell layers during morphogenetic processes such as gastrulation movement in *Xenopus* (Kusakabe and Nishida, 2004; Ossipova *et al.*, 2005) and vulva morphogenesis in *Caenorhabditis elegans* (Hurd and Kemphues, 2003). In a perhaps related process, Par1b-depleted MDCK cultures failed to execute the morphogenetic program by which they develop into tubular structures when embedded in collagen matrices (Cohen *et al.*, 2004).

The role of Par1 in early *Xenopus* morphogenesis has been attributed to its signaling in a branch of the wnt-signaling cascade that regulates convergent extension movements, most likely by modulating the actin cytoskeleton (Kusakabe and Nishida, 2004; Ossipova *et al.*, 2005). Dishevelled (Dvl), central to all branches of the wnt pathway, is an *in vitro* substrate of Par1 (Sun *et al.*, 2001) and the likely target of the kinase in this pathway. Although the Par1 phosphorylation sites in Dvl have not been identified, alanine substitutions of all eight serine/threonines within a 35-amino acid stretch to which the Par1 phosphorylation site(s) have been mapped (Sun *et al.*, 2001) inhibited the ability of Dvl to signal in the convergent–extension pathway (Ossipova *et al.*, 2005). In addition, a different *Xenopus* Par1 isoform stimulated the so-called canonical branch of wnt that regulates gene expression and determines cell fates during *Xenopus* development (Sun *et al.*, 2001; Ossipova *et al.*, 2005). Because the Par1 phosphomutant of Dvl was not defective in canonical wnt signaling, it is likely that Par1 regulates wnt cascades by more than one mechanism.

Although a wnt pathway akin to convergent–extension signaling has not been described in adult mammalian epithelial tissues, canonical wnt signaling is known to be central to the differentiation and homeostasis of epithelial cells.

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In the kidney, wnt-4 mediates the mesenchymal-to-epithelial transition that generates the various cell types of kidney tubule (Vainio, 2003). In differentiated cells, by contrast, wnt signaling often induces transformation (Smalley and Dale, 1999; Ilyas, 2005). In MDCK cells, recombinant expression of active LEF, a transcription factor stimulated by wnt in the canonical pathway, induces loss of epithelial characteristics known as epithelial-to-mesenchymal transition (EMT) (Kim *et al.*, 2002). A hallmark of EMT and of many epithelial derived tumors is the loss of E-cadherin-mediated adhesion, which allows epithelial cells to move out of the monolayer and invade the surrounding tissue (Thiery, 2002). The most common cause is a down-regulation of functional E-cadherin molecules on either the transcriptional or post-translational level by a variety of mechanisms (Khew-Goodall and Wadham, 2005). Work in tissue culture models has established that E-cadherin-mediated cell-cell adhesion provides an initial cue that triggers the execution of a program that results in epithelial polarization and includes cell shape changes associated with actin and microtubule (MT) reorganization, the formation of distinct luminal and basolateral surfaces and the establishment of tight junctions (Yeaman *et al.*, 1999; Nelson, 2003). These polarization events are based on the E-cadherin-dependent organization of an elaborate network of integral and peripheral membrane proteins that form scaffolding and signaling complexes at the lateral membrane, some of which associate directly or indirectly with the cytoplasmic domain of E-cadherin itself and with the cortical actin cytoskeleton (Jamora and Fuchs, 2002; Gumbiner, 2005).

Because Par1b promotes the E-cadherin-dependent columnarization and monolayer organization in polarizing MDCK cells, we tested whether the kinase regulates cell-cell adhesion and whether Par1b executes its role in epithelial polarization via the wnt cascade.

We report here that Par1b depletion by small interfering RNA (siRNA) reduced cell-cell adhesion, whereas Par1b overexpression had the opposite effect. Activation of canonical wnt signaling by recombinant expression of Dvl mimicked the phenotype of PAR1b depletion on cell adhesion, cell shape, monolayer organization, and tubulogenesis, whereas recombinant PAR1b blocked the effects of Dvl. Although this scenario suggested a role for Par1b in preventing EMT mediated by canonical wnt signaling, we determined that Dvl exerted the polarization defects independent of its downstream effectors in the canonical pathway. Moreover, neither Par1b knockdown nor Dvl expression decreased E-cadherin levels as reported for canonical wnt signaling. Instead, Par1b and Dvl acted antagonistically to regulate the association of E-cadherin with the actin cytoskeleton. After mapping the PAR1b phosphorylation sites in Dvl, we determined that PAR1b did not antagonize Dvl signaling by phosphorylating the wnt signaling molecule. We thus propose a model in which Par1b signaling antagonizes the wnt pathway to prevent epithelial transformation by regulating the association of E-cadherin with the lateral actin cortex.

MATERIALS AND METHODS

Cell Culture and Transfection

MDCK-Tet-OFF cells or MDCK-Tet-OFF cells stably expressing myc-tagged canine PAR1b were used as described previously (Cohen *et al.*, 2004). Recombinant PAR1b in MDCK-Par1b cells is expressed 10-fold above endogenous levels in the absence of doxycycline (dox) (Cohen *et al.*, 2004). MDCK-Tet-on cells (provided by T. Weimbs, University of California, Santa Barbara, CA) and MDCK-Tet-ON clones expressing the Par1b-siRNA construct were induced with 40 nM doxycycline for 48 h before being plated for the experiments described in Figures 1 and 5. For Ca^{2+} -switch experiments, cells were

cultured for 24 h in DMEM at subconfluence, plated at confluence in minimal essential medium for suspension culture (low Ca^{2+} medium; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS) dialyzed against phosphate-buffered saline, and switched back to DMEM (1.8 mM Ca^{2+}) after 10–16 h. Cells were analyzed 24–30 h after the Ca^{2+} -switch.

Collagen I overlay of subconfluent monolayers grown on collagen-coated coverslips was done as described previously (Cohen and Musch, 2003). Collagen-coated cells were analyzed 48 h after collagen overlay.

Cells were transiently transfected using AMAXA nucleofection technology (Amata, Biosystems, Gaithersburg, MD) (Cohen *et al.*, 2004). Fifteen micrograms of cDNA and 4×10^6 cells were used per transfection.

Plasmids

Construction and cloning of PAR1b has been described in Cohen *et al.* (2004). The kinase-dead mutant PAR1b-K49A was generated by site-directed mutagenesis and verified by sequencing. PAR1b and PAR1b-K49A were C-terminally tagged with the IgG-binding domain of protein A (Puig *et al.*, 2001) and cloned into pTRE2.

A Par1b siRNA construct similar to that described as pSUPER based in (Cohen *et al.*, 2004) was generated in the pSUPERIOR.puro vector (Oligo-engine, Seattle, WA) and expressed in stable MDCK-Tet-ON cell lines after selection in 2.5 $\mu\text{g}/\text{ml}$ puromycin.

Mouse Dvl1 cDNA, provided by Dr. Sussman (University of Maryland, College Park, MD) was cloned into pCMV-Tag1 vector (Stratagene, La Jolla, CA) upstream of the FLAG-tag. Then, Dvl1 with the C-terminal FLAG-tag was cloned into SacII/HindIII sites of pTRE2 vector for expression of the protein in MDCK-Tet-OFF cells. The Dvl1DEP construct encompassing amino acids 1–373 was cloned into pTRE2 in a similar manner. The Dvl fragment comprising amino acids 200–250 linked to an N-terminal FLAG-tag were inserted into pTRE2 by PCR cloning.

DvlM glutathione S-transferase (GST)-fusion protein was obtained by subcloning residues 173–395 of Dvl1 into EcoRI/Xho I sites of pGEX4T1 vector (GE Healthcare Life Sciences, Piscataway, NJ). All DvlM mutants were created by site-directed PCR mutagenesis (Stratagene) and verified by sequencing. GST-fusion proteins were produced, purified, and their protein concentrations were estimated as described previously (Rossi *et al.*, 1997).

TOPFLASH (firefly luciferase reporter with 3 LEF-binding domains), FOPFLASH (LEF-binding defect reporter) (Molenaar *et al.*, 1996), and pRLTK (Renilla luciferase reporter) were obtained from Dr. Bert Vogelstein (Johns Hopkins School of Medicine, Baltimore, MD), and the β -catenin constructs $\Delta\text{N}90$ and β -catenin-S37A were from Louise Howe and Anthony Brown (Cornell University Medical College, New York, NY).

Antibodies

Antibodies used were as follows: anti-myc (clone 9E10; Santa Cruz Biotechnology, Santa Cruz, CA); anti-gp135 (mouse, clone 3F21D8; provided by G. Ojakian, SUNY Downstate Medical Center, Brooklyn, NY); anti-ZO1 (rat; Chemicon International, Temecula, CA); anti-E-cadherin (clone rr1; Gumbiner and Simons, 1986), obtained from the Hybridoma Bank (University of Iowa, Iowa City, IA); anti- β -tubulin (rat, clone TUB 2.1; Sigma-Aldrich, St. Louis, MO); anti- β -catenin (rabbit; Sigma-Aldrich); anti-c-Jun-NH₂-terminal kinase (JNK) (clone F-3; Santa Cruz Biotechnology); phospho-specific c-Jun-S63 (Cell Signaling Technology, Beverly, MA); anti-Dvl1 (clone 3F12; Santa Cruz Biotechnology); and TOPRO nuclear stain (Invitrogen).

Hanging Drop Assays

For the hanging drop assays, 20,000 single cells were suspended in 35- μl drops of DMEM/10% FBS from the lid of 24-well cluster for 16 h. The corresponding bottom wells contained water to maintain humidity. The drops were pipetted five times up and down with a 200- μl standard tip, fixed with 2% glutaraldehyde, and aliquots were spread on coverslips. Images of six random fields from five samples were taken with a 4 $\times$ Achromplan lens (numerical aperture [NA] 0.10) on an inverted Axiovert (Carl Zeiss, Thornwood, NY) for each sample. The area of cell clusters was determined using the Integrated Morphometric Analysis Tool of MetaMorph (Molecular Devices, Sunnyvale, CA). The area occupied by single cells was measured in parallel to estimate the amount of cells per area unit.

Immunofluorescence and Immunoblots

Immunofluorescence. Cells were fixed with ice-cold 2% paraformaldehyde and permeabilized with 0.2% Triton X-100 (Tx100). For MT analysis, cells were extracted for 30 s at 37°C with PEM buffer (100 mM PIPES, pH 6.8, 1 mM EGTA, and 1 mM MgCl_2) supplemented with 0.5% Tx100, and then fixed with ice-cold methanol. Fixed cells were incubated for 1 h in 1% bovine serum albumin (BSA), stained with primary antibodies for 1 h, exposed to secondary antibodies for 45 min, and mounted using VECTASHIELD (Vector Laboratories, Burlingame, CA).

Confocal microscopy was performed with a model SP2 (Leica Microsystems, Deerfield, IL) using a 63 $\times$ oil objective (NA 1.4). Individual confocal x-y and x-z sections are presented. All samples from one experiment were

imaged with the same laser settings and detector gain. Images were processed with Adobe Photoshop Adobe Systems, Mountain View, CA). Cell height was determined from confocal x-z-views of phalloidin-labeled cells in 10 random fields using Leica software. Compaction was calculated from the same images by counting cells. All x-z-views are taken with the same zoom and span a length of 125 μm . Numbers are derived from two experiments and are presented with standard deviations.

Immunoblots. Cells grown on 2- or 4-cm² plastic surfaces were washed with Hank's balanced salt solution and lysed in 50 or 100 μl of SDS sample buffer or extracted with actin extraction buffer (Cramer and Mitchison, 1995) [138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 0.32 M sucrose, 10 mM 2-(N-morpholino)ethanesulfonic acid, pH 6.1, 0.5% Tx100, and 2 mM phenylmethylsulfonyl fluoride] for 10 min on ice before SDS lysis. Immunoblots were developed with ¹²⁵I-protein A, and the signal was detected and quantified by PhosphorImager analysis (Typhoon Trio; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom).

Luciferase Reporter Assays

MDCK cells were transfected with 1 μg of pRLTK (*Renilla* luciferase), 2 μg TOPFLASH (firefly luciferase reporter under control of TCF/LEF-responsive elements) or FOPFLASH (control for TOPFLASH) and Dvl, β -catenin, or vector constructs as indicated. Cells were cultured for 24 h in DMEM, lysed, and assayed for luciferase activities in a luminometer using the dual-luciferase reporter assay system (Promega). The net reporter activity was calculated as the ratio of firefly luciferase reporter activity to *Renilla* activity. Data are from four experiments with quadruplicate samples. SEs are indicated.

In Vitro Kinase Assays

Dvl Phosphorylation. MDCK cells expressing myc-tagged PAR1b and control MDCK-Tet-OFF cells were cultured in the absence of doxycycline to confluence, were homogenized by nitrogen cavitation in HB buffer (10 mM HEPES/KOH, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol [DTT], 1 $\times$ phosphatase inhibitors, and 2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride [AEBSEF]). Postnuclear supernatants were adjusted to 150 mM NaCl, 4 mM EDTA, 1% Tx100, and 0.2% BSA, extracted on ice for 30 min, and incubated with pansorbin (Calbiochem, San Diego, CA) for 45 min. Lysates were subjected to immunoprecipitation with myc-bound protein A for 3–4 h. Immune complexes bound to protein A were incubated with 12–15 μg of purified recombinant Dvl protein in KB buffer (40 mM HEPES, pH 7.2, 5 mM MgCl₂, 2 mM EGTA, 0.1% Tx100) supplemented with 0.2 mM DTT, 1 mM ATP, protease inhibitors (10 mg/ml leupeptin, pepstatin, and antipain), 2 mM AEBSEF, and 1 $\times$ phosphatase inhibitor cocktail I (Sigma-Aldrich), 1 mM Na-orthovanadate and 1 μl of [γ -³²P]ATP (10 μCi). Kinase reactions (40 μl) were incubated at 30°C for 30 min, pelleted by centrifugation, and supernatants were boiled in an equal volume of a sample buffer. Samples were subjected to SDS-PAGE, and gels were dried and exposed to film. For the experiments in Supplemental Figure S1, cell lines expressing protein A-tagged PAR1b and K49A-PAR1b were used, and the recombinant protein was isolated on IgG-Sepharose.

JNK Activity Assay. Cells were lysed with 1% Tx100 in 20 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA, and 0.2% BSA, supplemented with 2 mM AEBSEF 10 $\mu\text{g}/\text{ml}$ each leupeptin, pepstatin, antipain, and 5 mM NaF, 1 mM vanadate, 5 mM Na-pyrophosphate, and 1 $\times$ phosphatase inhibitor cocktail I (Sigma-Aldrich). 250 μg of lysate was immunoprecipitated with 2 μg of JNK antibody, and the immobilized immunocomplexes were incubated with 2.5 μg of recombinant c-Jun (Santa Cruz Biotechnology) in KB buffer and 1 mM ATP for 30 min at 30°C. After the kinase reaction, the supernatants and pellets were analyzed in immunoblots for phospho-c-Jun and JNK, respectively. Data presented are representative of two experiments.

RESULTS

Par1b Promotes Cell–Cell Adhesion

Contact-naïve MDCK cells have a fibroblastic cell shape, exhibit a radial MT array that emanates from the perinuclear area, and lack tight junctions. On cell–cell contact, E-cadherin-mediated adhesion triggers cell compaction and columnarization associated with the growth of the lateral membrane and with microfilament reorganization, tight junctions form at the cell apex and MTs rearrange into vertical arrays that run along the apico-basolateral polarity axis. Reduction of Par1b levels inhibited or delayed all of the above-mentioned polarization events (Cohen *et al.*, 2004; Suzuki *et al.*, 2004). In addition, transient expression of a kinase-deficient form of Par1b caused cell rounding and expulsion of the transfected cells

from the monolayer (Bohm *et al.*, 1997). These observations prompted us to investigate whether Par1b directly regulates cell–cell adhesion.

We generated stable cell lines that express a previously characterized plasmid-based Par1b-siRNA construct (Cohen *et al.*, 2004) under a tetracycline-inducible promoter (Par1b-KO TET-ON MDCK, clones 12 and A; Figure 1A) and compared the adhesive properties of the parental cells with those of two Par1b-KO clones cultured in the presence and absence of doxycycline. We used the hanging drop assay that measures the size of cell clusters that form in suspended drops overnight and that resist titration with a micropipette tip (Redfield *et al.*, 1997). Cluster sizes were evaluated from 4 $\times$ phase images by morphometric image analysis (see *Materials and Methods*) and classified according to their area. Figure 1B indicates that Par1b depletion (Par1b-KO+dox) abolishes large clusters (10^5 – $2 \times 10^4 \mu\text{m}^2$) corresponding to $\sim$ 100–600 cells and reduces to about one-half the clusters measuring 2×10^4 – $1 \times 10^4 \mu\text{m}^2$ or $\sim$ 55–100 cells. An $\sim$ 10-fold doxycycline-dependent overexpression of Par1b (Cohen *et al.*, 2004), in contrast, induced clusters larger than $10^5 \mu\text{m}^2$ that were not observed under control conditions (Figure 1B). Together, our data indicate that Par1b promotes cell–cell adhesion, a feature characteristic of a tumor suppressor.

Dvl Phenocopies Par1b Depletion and Par1b Overexpression Antagonizes Dvl-mediated Disruption of Cell–Cell Adhesion and Polarization

Because loss of cell–cell adhesion has been associated with aberrant wnt signaling and Dvl has been identified as a Par1 substrate we set out to determine whether Dvl was the relevant target of Par1b in MDCK cell adhesion and polarization.

Dvl overexpression in various cell culture models has been shown to mimic the activation of the canonical- and the convergent–extension/planar cell polarity (PCP)–wnt pathway that result in the activation of transcription factors of the TCF/LEF family and of the kinase JNK and its target c-Jun, respectively (Li *et al.*, 1999; Sun *et al.*, 2001) (see Figure 2A for details of the pathways). In agreement with these earlier findings, we detected a three- to fivefold stimulation of TCF/LEF1 activity in a reporter gene assay (Figure 2C, TOPFLASH assay; Molenaar *et al.*, 1996) and a threefold stimulation of JNK activity (Figure 2D) in Dvl1-overexpressing MDCK cells. We established therefore that recombinant Dvl activates both wnt pathways in our model system.

After validating its role in wnt signaling, we next tested whether recombinant Dvl mimics or modulates the cell–cell adhesion and morphogenetic phenotype observed in MDCK cells with reduced PAR1b kinase activity.

When analyzed in hanging drop assays, Dvl overexpression in control cells reduced by 4.4-fold the number of large adhesion clusters with 100 and more cells (Figure 3 A, top, and table), a similar phenotype as observed for Par1b depletion.

We next analyzed the effect of Dvl1 overexpression on the development of MDCK polarity in control cells using two different models—the collagen overlay assay (a 3-dimensional culture model) and the Ca²⁺-switch approach (a 2-dimensional culture model). In the collagen overlay assay, MDCK monolayers cultured on collagen substrates undergo tubulogenesis when overlaid with additional collagen at their apex (Hall *et al.*, 1982). Tubule formation involves a partial epithelial-to-mesenchymal transition during which the luminal surface is eliminated and cell adhesion mechanisms are relaxed. Cells then migrate to rearrange into two cell layers before they subsequently reestablish polarity with

A dox-inducible Par1b-KO cell lines

B cell-cell adhesion in hanging-drop assays

Cell line	Aggregates per field			
	$>10^5$ μm^2	$<10^5 -$ $2 \times 10^4 \mu\text{m}^2$	$<2 \times 10^4 -$ $1 \times 10^4 \mu\text{m}^2$	$<10^4 -$ $3.5 \times 10^3 \mu\text{m}^2$
MDCK-TET ON dox-induced				
TET-ON - dox	0	2.7	5.8	5.0
TET-ON + dox	0	2.0	6.0	5.0
Par1b-KO cl.12 - dox	0	1.0	4.4	5.2
Par1b-KO cl.12 + dox	0	0	1.5	6.3
Par1b-KO cl.A - dox	0	1.8	5.2	6.0
Par1b-KO cl.A + dox	0	0.15	3.0	11.0

Cell line	Aggregates per field			
	$>10^5$ μm^2	$<10^5 -$ $2 \times 10^4 \mu\text{m}^2$	$<2 \times 10^4 -$ $1 \times 10^4 \mu\text{m}^2$	$<10^4 -$ $3.5 \times 10^3 \mu\text{m}^2$
MDCK-TET OFF dox repressed				
Par1b cl.12 + dox	0	2.5	2.0	1.8
Par1b cl.12 - dox	0.75	0	0.6	1.0
Par1b cl.24 + dox	0	2.0	1.5	1.5
Par1b cl.24 - dox	0.55	0.5	1.0	0.5

Figure 1. Par1b regulates cell–cell adhesion. (A) Doxycycline-inducible Par1b-KO cell lines. Parental TET-ON cells and two Par1b-KO clones (cl.12 and cl.A) were cultured in the presence (+dox) or absence (–dox) of doxycycline as described in *Materials and Methods*. An antibody that recognizes all Par1 isoforms was used to probe for Par1 expression in equal amounts of SDS lysates. Par1b-specific bands are indicated by asterisks. (B) Cell–cell adhesion in hanging drop assays. Parental MDCK-TET-ON cells and Par1b-KO cells cl.12 and A (top) or Par1b-MDCK-TET-OFF cells cl.12 and 24 (bottom) were cultured in the presence or absence of dox. Cell–cell adhesion upon suspension in hanging drops was determined from 4× wide-field images (right) as described in *Materials and Methods*. The areas of cell clusters in six random fields taken from five different hanging drops were determined for each culture condition and classified as indicated. An area of $10^5 \mu\text{m}^2$ corresponds to ~ 600 cells, $2 \times 10^4 \mu\text{m}^2$ corresponds to ~ 100 cells, $10^4 \mu\text{m}^2$ corresponds to ~ 55 cells, and $3.5 \times 10^3 \mu\text{m}^2$ corresponds to 20 cells. Presented are the average clusters per field for each classifier from one representative experiment out of two (Par1b-MDCK) or three (Par1b-KO) independent experiments. Bars, 300 μm .

de novo formation of an apical domain that lines the central lumen of an epithelial tubule as indicated by labeling of the luminal membrane with antibodies to a well characterized marker gp135/podocalyxin (Figure 3A, collagen overlay, vector). We had previously reported that inhibition of PAR1b by siRNA-mediated knockdown (PAR1b-KO) or by expression of kinase-deficient PAR1b (KN-PAR1b) inhibited directed cell migration and lumen formation in this assay (Cohen *et al.*, 2004). Figure 3A (collagen overlay panel, Dvl), depicts that Dvl-overexpressing MDCK cells exhibit a similar phenotype: cells remained monolayered after collagen overlay and lacked a luminal surface.

The Ca^{2+} -switch assay measures development of polarity in MDCK cultures grown on porous filter substrates. Confluent MDCK monolayers that are prevented from establishing E-cadherin-mediated cell–cell contacts by low (micromolar) levels of Ca^{2+} in the culture medium, undergo rapid synchronous polarization when they are switched to normal Ca^{2+} medium (Gonzalez-Mariscal *et al.*, 1990). Twenty-four hours after Ca^{2+} -switch, control MDCK cells have compacted and developed a columnar architecture, resulting from elongation of the lateral membrane domain that becomes separated from the luminal domain by tight junctions (Figure 3A, Ca^{2+} -switch panel, vector). MDCK cells also reorganized their MT network from a radial, centrosome-focused array into a noncentrosomal network with MTs running along the apico-basolateral polarity axis (Figure 3A, bottom, vector). Expression of Dvl1 prevented MT reorga-

nization after Ca^{2+} -switch and cells maintained a radial MT array (Figure 3A, bottom, Dvl). The height of Dvl1 cells was only $5 \pm 2 \mu\text{m}$ compared with $14 \pm 2 \mu\text{m}$ in control cells as evident in x-z views of cells labeled with phalloidin (Figure 3A, Ca^{2+} -switch panel), and cells were notably less compacted. We measured compaction by counting the number of cells along a horizontal line of 125 μm in confocal x-z views as those in Figure 3. Whereas control cells packed on average 10 ± 3 cells along the distance, Dvl1-expressing cells featured only 6 ± 1 cells.

The defects in lumen formation, compaction, elongation, and in MT reorganization are all hallmark of the PAR1b-KO phenotype. As previously observed for PAR1b-KO (Cohen *et al.*, 2004), Dvl1 overexpression did not prevent the formation of two distinct surface domains and did not cause missorting of luminal and basolateral proteins in two-dimensional monolayer cultures (our unpublished data).

Collectively, our data indicate that Dvl1 overexpression mimics the phenotype of PAR1b inhibition in polarizing MDCK cells. Thus, we next determined whether recombinant PAR1b attenuates the Dvl phenotype on cell–cell adhesion and morphology.

Because we achieved robust Dvl expression only when the cDNA was placed under a tetracycline-dependent promoter, we compared the phenotype of recombinant Dvl in the parental MDCK-Tet-OFF cells (control MDCK in Figure 3) with that in two clones of Par1b-MDCK cells (clones 12 and 24) cultured under inducible conditions (without doxycycline).

Figure 2. Dvl1 promotes wnt signaling in MDCK cells and is phosphorylated by Par1b on two sites in vitro. (A) Models for Dvl in the canonical and planar polarity wnt pathways. The wnt signal is transduced via the membrane receptor Frizzled and its coreceptor LRP to Dvl, a soluble protein with three functional domains. Canonical pathway: Dvl inhibits GSK3 β . GSK3 β functions in a destruction complex with Axin and APC to phosphorylate β -catenin and targets it for degradation. β -catenin that accumulates in the nucleus acts as co-transcription factor for TCF/LEF; requires all three domains of Dvl. Planar polarity pathway: Dvl activates JNK, presumably via rho-GTPases, which leads to c-Jun-dependent gene transcription and to cytoskeletal rearrangements; requires the Dvl-DEP domain. (B) PAR1b phosphorylates Dvl1 on Ser 236 and Ser 243 in vitro. Immobilized immune complexes containing MDCK-derived PAR1b (+PAR1b-myc) or IgG (control) were incubated with purified recombinant DvlM proteins (amino acids 173–395) and [γ - 32 P]ATP as described in *Materials and Methods*. Evolutionary conserved Ser/Thr residues in the target region (amino acids 213–248) were sequentially substituted to alanine by site-directed mutagenesis. Substitution of both Ser 236 and Ser 243 to alanine abolished PAR1b-dependent phosphorylation. (C) Dvl promotes TCF/LEF activation. TOPFLASH or FOPFLASH control assays of MDCK cells cotransfected with either 12 μ g of vector DNA or WT-Dvl plasmids. Normalized luciferase activity is expressed as -fold increase over the baseline activity in vector-transfected cells. (D) Dvl promotes JNK activation. Cells were transfected with either vector DNA or WT-Dvl cDNA as described in C and analyzed for JNK activity with c-Jun as substrate as described in *Materials and Methods*. Duplicate samples are presented. Top, phospho-c-Jun. Bottom, JNK immunoprecipitation.

Compared with Dvl-expressing control MDCK cells, Dvl-expressing Par1b-MDCK cells formed more than twice as many large cell clusters, measuring 10^5 – 2×10^4 μ m 2 , in the hanging drop assay, indicating that Par1b attenuated the effect of Dvl on cell–cell adhesion (compare Figure 3, A and B, cell–cell adhesion panels and table). Likewise, coexpression of Par1b with Dvl in Ca $^{2+}$ -switch assays rescued the morphogenetic phenotype caused by overexpression of Dvl alone (shown for MDCK-Par1b clone 12 in Figure 3B, similar results were obtained with clone 24). Cells exhibited strong gp135 labeling at the apex and the cell height increased from 5 ± 2 to 9 ± 3 μ m. Dvl-expressing PAR1b-MDCK cells were also more compacted than the corresponding Dvl-expressing control cells with an average of 9 ± 1.5 cells versus 6 ± 1 cells in individual x-z sections. We also observed a rescue, albeit only partial, of the Dvl phenotype by PAR1b in the tubulogenesis assay. Dvl-PAR1b cells developed multiple small lumina upon collagen overlay compared with the complete absence of a luminal domain in Dvl cells on one hand and to the central lumen in control cells on the other hand. These data indicate that PAR1b attenuates Dvl signaling in cell–cell adhesion and in the polarization of MDCK cells.

MDCK-PAR1b Phosphorylates Dvl1 on Two Sites In Vitro

The antagonistic behavior of PAR1b and Dvl in our functional assays raised the possibility that the kinase inhibits Dvl by direct phosphorylation. To test this hypothesis, we first mapped the phosphorylation sites of Par1b in Dvl.

Dvl is a soluble molecule composed of three functionally separable domains, DIX, PDZ, and DEP. Sun *et al.* (2001) had narrowed the region that contains the PAR1 phosphorylation sites to a stretch of 35 aa in the PDZ region that is rich in serines and threonines. We used systematic site-directed mutagenesis within a mouse Dvl1 fragment (DvlM aa 173–395) that covered the Par1 phosphorylation region and determined with in vitro kinase assays that simultaneous mutation of two serine residues, Ser236 and Ser243, to alanine completely abrogated the PAR1b-mediated phosphorylation (Figure 2B). We used Par1b immunoprecipitated from MDCK-Par1b cells as source of the kinase in these assays. To rule out that a coprecipitating kinase was responsible for the phosphorylation, we performed parallel assays with a kinase-dead form of PAR1b (PAR1b-K49A), which caused 10-fold reduced phosphorylation levels of DvlM-WT, DvlM-A236, and DvlMA243, indicating that Dvl phosphorylation at both sites is dependent on PAR1b activity (Supplemental Figure S1).

The Dvl Phenotype Is Independent of Par1-mediated Dvl Phosphorylation

We were now able to compare the morphogenetic effects of wild-type (WT) Dvl with that of a Dvl-mutant in which the Par1 phosphorylation sites had been abolished (DvlS235,243A). When expressed in control cells, DvlS236,243A caused a similar phenotype as the WT-protein in cell–cell adhesion, Ca $^{2+}$ -

Figure 3. PAR1b and Dvl function antagonistically to regulate MDCK polarization. (A) Dvl and Dvl S236,243A expression reduce cell–cell adhesion, block lumen formation, compaction, and MT reorganization. Control MDCK cells transiently transfected with vector DNA, Dvl wild type, or DvlS236,243S were subjected to cell–cell adhesion assays (top), collagen overlay (middle), or Ca²⁺-switch assays (bottom). Cells were labeled for the indicated markers. Presented are 4× wide-field images in the top panel, confocal x-z views in the two middle panels, and a confocal x-y section taken through the center of the cells in the MT panels (bottom). Note the cross section through vertical MTs in the control cells that is absent in the Dvl samples. (B) PAR1b blocks the Dvl and DvlS236,243A phenotypes. PAR1b-MDCK cells were transfected, cultured, and processed as the control cells described in A. Bars, 300 μm (cell–cell adhesion panels) and 10 μm (all other panels). Table shows results from a hanging drop assay performed as described in *Materials and Methods* and in Figure 1. Data are representative of three (MDCK control) and two (MDCK-Par1b) independent experiments.

switch- and tubulogenesis assays (Figure 3B and table). Importantly, recombinant PAR1b rescued the DvlS236,243A phenotype to the same extent as that of Dvl-WT in all assays (Figure 3B and table). The average cell height increases from 4.8 ± 2 to 9.8 ± 3 μm and the compaction index from 6.5 ± 1 to 9.5 ± 1 cells/125 μm. These data suggest that the antagonistic function of Dvl and PAR1b does not involve PAR1b-mediated Dvl phosphorylation. In line with this conclusion, we did not observe any increase in Dvl phosphorylation by PAR1b overexpression in vivo (our unpublished data).

Dvl Regulates MDCK Polarity Independently of the Canonical or Planar Polarity-wnt Pathways

Our data suggest that PAR1b either intersects with or supplants a Dvl-signaling pathway that regulates MDCK cell–cell adhesion and morphology. To delineate this pathway, we analyzed the signaling downstream of Dvl in the canonical pathway. By inhibiting the kinase glycogen synthase kinase 3β (GSK3β), Dvl prevents a destabilizing phosphorylation of β-catenin and allows it to act as cotranscription factor in the nucleus (for review, see Logan and Nusse, 2004; Figure 2A).

A polarization of control MDCK **B TCF/LEF activation** **C Hanging drop assay**

transfections	Aggregates per field		
MDCK-TET-OFF - dox	<10 ⁵ - 2x10 ⁴ μm ²	<2x10 ⁴ - 1x10 ⁴ μm ²	<10 ⁴ - 3.5x10 ³ μm ²
MDCK+vector*	3.1	3.5	6.0
+Dvl*	0.7	2.5	8.6
+βcateninΔN90	3.4	2.0	4.5
+DvlΔDEP	0.5	2.6	6.2

* Data from Fig.3

Figure 4. Dvl regulates MDCK polarity independent of canonical wnt signaling. Control cells were either mock transfected (vector) or transfected with 12 μg of cDNA encoding DvlΔDEP or myc-tagged β-cateninΔN90 in the presence (B) or absence (A and C) of the TOPFLASH reporter plasmids. (A) DvlΔDEP but not β-cateninΔN90 mimic the Dvl phenotype. MDCK cells were subjected to cell-cell adhesion assays, collagen overlay, or Ca²⁺-switch and analyzed for tubulogenesis (collagen overlay, x-z view), lumen formation, cell compaction, and shape (middle, x-z view) and MT organization (bottom, x-y view) as described in Figure 3. Graph shows -fold stimulation of TOPFLASH-dependent luciferase activity by Dvl, DvlΔDEP, and β-cateninΔN90. The value for the Dvl-bar is derived from Figure 2C. Image shows myc-labeling of vector and β-cateninΔN90 transfected cells; x-y view through the cell center indicates homogenous expression of recombinant β-catenin within the cell population. Bars, 300 μm (cell-cell adhesion panels) and 10 μm (all other panels). (C) Hanging drop assay. Assays were performed as described in Figure 1 and *Materials and Methods*. Data are representative of three independent experiments.

Overexpression of a β-catenin mutant resistant to GSK3β phosphorylation (β-catenin ΔN90) stimulated TCF/LEF-mediated gene transcription to a comparable extent as Dvl expression (Figure 4B), yet it failed to cause the adhesion or morphogenic phenotypes observed with Dvl expression (Figure 4, A

and C, compare with Dvl panels in Figure 3A). We ascertained that myc-tagged β-cateninΔN90 was expressed homogeneously in the MDCK culture and hence ruled out that the TOPFLASH signal resulted from expression of recombinant β-catenin in only a few cells that would contribute little

A total E-cadherin **B Tx100 resistant E-Cadherin**

Figure 5. PAR1b and Dvl regulate the association of E-cadherin with microfilaments. (A) Total E-cadherin levels. MDCK-TET-OFF cells transiently expressing the indicated recombinant proteins (lanes 1–5) or parental TET-ON (control), Par1b-KO cl12, and Par1b-KO cl.A cells induced for 2 d with dox (lanes 6–8) were plated at confluence for 24 h, and equal amounts of SDS lysates were probed for E-cadherin in immunoblots. (B) Tx100-resistant E-cadherin. Total SDS lysates (1 and 2) and SDS lysates of Tx100-extracted cells (3 and 4) were probed for the presence of E-cadherin in quantitative immunoblots. Two times as much lysate from samples 3 and 4 was loaded as from 1 and 2; Graphs express the ratio of Tx100-resistant E-cadherin (3 and 4) to total E-cadherin (1 and 2) for each cell culture condition from three independent experiments with SEs. Note that the total protein levels are not adjusted between the different cell culture conditions. Top, parental TET-ON cells or Par1b-KO clones 12 and A were either induced (+dox) or remained uninduced (–dox). The small decrease in Tx100-resistant E-cadherin in the uninduced Par1b-KO clones (–dox) compared with the parental cells is likely due to leakiness of the expression system, because both Par1-KO clones express slightly less Par1 than the parental cells even before doxycycline induction. Bottom, parental TET-OFF cells (control MDCK) transfected with either vector, Dvl, or βcatΔN90 cDNA and Par1b-MDCK cells transfected with either vector or Dvl cDNA were cultured under inducible conditions (–dox). Note that

Par1b-KO and Dvl expression do not decrease the total Tx100-resistant actin pool (Supplemental Figure S3).

to the overall morphogenesis of the cell culture (Figure 4B). We conclude that β-catenin in its capacity as transcription activator in the canonical wnt pathway is not involved in the polarization defects caused by Dvl expression.

We also evaluated whether Dvl signaling via the JNK pathway was required for the Dvl-mediated polarity phenotype. For this purpose, we expressed a Dvl deletion mutant lacking the C-terminal DEP domain that is obligate for PCP signaling in flies and mammals (Axelrod *et al.*, 1998; Li *et al.*, 1999). Surprisingly, DvlΔDEP caused a similar inhibition of cell–cell adhesion, cell compaction and lumen formation as the wild-type protein (Figure 4, A and C). The DEP mutant was also deficient in stimulating TCF/LEF transcription activation (Li *et al.*, 1999; Figure 4B), indicating that neither the JNK/PCP pathway nor the canonical pathway is responsible for the Dvl-mediated phenotype. We conclude that the morphogenetic effects of Dvl are mediated by neither of the two established branches of the wnt cascade.

In *Drosophila* embryos, Par1 has been described as a Dvl-interacting kinase and both proteins were efficiently coimmunoprecipitated from embryonic extracts (Sun *et al.*, 2001). We were unable to find a similar interaction in MDCK cell lysates, even when Par1b and Dvl were overexpressed (our unpublished data). We nevertheless tested the possibility that recombinant Dvl mimicked Par1 depletion by binding the kinase and titrating it away from its substrates. Our first approach was to activate the wnt pathway with the wnt ligand rather than with recombinant Dvl. However, only coculture with wnt-1 secreting fibroblasts but neither recombinant wnt-1 expression nor wnt-conditioned medium ap-

plied to both surface domains induced TCF/LEF activation in MDCK cells (our unpublished data). Because coculture with control fibroblasts altered MDCK monolayer organization by itself, we were unable to assess the morphogenetic effects of wnt. As an alternative approach, we expressed a FLAG-tagged Dvl peptide encompassing the Par1b-binding domain (Dvl aa 200–250). This peptide blocked the stimulation by Par1 of canonical wnt signaling in Chinese hamster ovary cells (Sun *et al.*, 2001). At comparable expression levels as FLAG-tagged full-length Dvl (Supplemental Figure S2B), the Dvl peptide did not exhibit any phenotype in any of the morphogenetic assays or when cell–cell adhesion was analyzed (Supplemental Figure S2A). It is unlikely therefore, that recombinant Dvl simply exerts a nonspecific effect on Par1b signaling by interacting with the kinase.

Par1b Antagonizes Dvl by Promoting the Interaction of E-Cadherin with the Actin Cytoskeleton

Our previous observations and preliminary experiments indicated that Par1b does not reduce E-cadherin levels (Figure 5A) or disrupt its polarized distribution at the lateral surface (Cohen *et al.*, 2004). This left open the possibility that Par1b regulates the formation of E-cadherin protein complexes at the cortex. E-cadherin function is critically dependent on its association with microfilaments that form a circumferential actin belt at the level of the adhesion junctions and connect neighboring cells (Quinlan and Hyatt, 1999). The mechanism of E-cadherin–microfilament interactions is not yet understood, although it is likely mediated through actin-binding proteins that are present at adhesion junctions (Yamada *et*

al., 2005). When analyzed 36h upon plating at confluence, $65 \pm 5\%$ of E-cadherin in control cells (parental TET-ON cells+dox) is resistant to extraction with Tx100, a measure of its association with the actin cytoskeleton. By contrast, only $28 \pm 5.6\%$ and $31 \pm 3.5\%$ of E-cadherin in either Par1b-KO clone is Tx100 resistant when induced with doxycycline (Figure 5B, top).

As seen for the cell adhesion and polarization assays, Dvl expression mimicked the effect of Par1b depletion and reduced the Tx100-resistant pool of E-cadherin (to $20 \pm 6\%$ in Dvl cells from $53 \pm 5\%$ in controls), whereas β -catenin did not cause any defect in E-cadherin-microfilament association ($58 \pm 3.6\%$).

Importantly, Par1b attenuated the effect of Dvl on E-cadherin insolubility (Figure 5B, bottom; $42 \pm 4.6\%$ Tx100-resistant E-cadherin in Dvl-expressing Par1b cells). Thus, regulating the interactions of E-cadherin with microfilaments might represent an underlying mechanism for the antagonistic roles of Par1b and Dvl in cell-cell adhesion and MDCK polarization.

DISCUSSION

Observations that altered expression of Par1 isoforms is associated with at least two types of cancer, together with recent findings that Par1 promotes the establishment of organized epithelial monolayers, has prompted speculations that Par1 might have tumor suppressor functions.

We have demonstrated here two features characteristic of a tumor suppressor for the mammalian isoform Par1b: the kinase promotes cell-cell adhesion and attenuates the effect of wnt signaling, induced by Dvl expression, which causes transformation of epithelial cells (Uthoff *et al.*, 2001; Nagahata *et al.*, 2003; Okino *et al.*, 2003; Uematsu *et al.*, 2003). As one underlying mechanism for these Par1b activities, we have determined that the kinase promotes resistance of E-cadherin to extraction with nonionic detergents, a common assay for cytoskeletal association (Hirano *et al.*, 1987; Nagafuchi and Takeichi, 1988; Shore and Nelson, 1991). The association of the E-cadherin cytoplasmic domain with the actin cytoskeleton is critical for E-cadherin function, because pharmacological agents that disrupt the actin cytoskeleton cause a rapid loss of cell-cell adhesion (for review, see Bershadsky, 2004). Because E-cadherin function is central to many aspects of epithelial morphogenesis and polarization, it is conceivable that compromised E-cadherin-mediated adhesion in Par1b-depleted cells also contributes to the defects in columnarization, compaction, apical surface formation, and MT organization that we have reported previously. We do not yet have insight into the molecular mechanism underlying the altered Tx100 extractability of E-cadherin. Par1b depletion and Dvl overexpression could affect the assembly and dynamics of actin filaments at adhesion junctions that are thought to be under the control of the microfilament-nucleator complex Arp2/3 and formin-1. Alternatively, the interaction of E-cadherin with microfilaments itself could be compromised. Recent work by the Nelson and Weis laboratories (Yamada *et al.*, 2005; Drees *et al.*, 2005) has deconstructed the longstanding assumption of an E-cadherin- β -catenin- α -catenin link to the actin cytoskeleton and thus the mechanism for the E-cadherin-microfilament interaction is currently not understood (for review, see Gates and Peifer, 2005).

Our findings that recombinant Dvl mimicked all aspects of the Par1b-KO phenotype, whereas overexpression of PAR1b rescued the Dvl-induced polarity phenotype, made Dvl a candidate PAR1b substrate for polarity development

in MDCK cells. After mapping the PAR1b phosphorylation sites in Dvl to serines 236 and 243, we determined, however, that Dvl phosphorylation is not responsible for the effect of PAR1b on Dvl signaling because PAR1b rescued the polarity phenotype caused by a nonphosphorylatable Dvl mutant as efficiently as it antagonized signaling by wild-type Dvl.

Our findings are in agreement with a recent report that Par1 can regulate wnt signaling in the *Xenopus* embryo by mechanisms other than Dvl phosphorylation (Ossipova *et al.*, 2005). The antagonistic relationship between Dvl and PAR1b that we observed differs, however, from the synergistic effect of Par1 and Dvl on TCF/LEF activity reported for *Drosophila* and *Xenopus* embryos and for cultured mammalian nonepithelial cells (Sun *et al.*, 2001; Spicer *et al.*, 2003; Ossipova *et al.*, 2005). We found that PAR1b overexpression attenuated Dvl signaling in MDCK polarization without significantly altering TCF/LEF activity either by itself or when coexpressed with Dvl (our unpublished data). These differences are likely due to context-dependent regulation of wnt signals that are common and in fact expected given the plethora of effects attributed to the wnt-signaling cascade. In a recent example, the serine/threonine kinase LKB1 was shown to enhance or inhibit canonical wnt signaling in different mammalian cell types (Ossipova *et al.*, 2003; Spicer *et al.*, 2003).

We have not yet determined whether Par1b directly intersects with the wnt-signaling cascade in MDCK cells, or, alternatively, supersedes the inhibitory effect of Dvl on polarization by regulating an independent signaling pathway. In either case, our data suggest that the effect of Dvl is independent of the well characterized canonical and PCP/JNK wnt pathways. The canonical pathway that leads to β -catenin stabilization and promotes its function as cotranscription factor in LEF/TCF-mediated gene transcription has been implicated in EMT (Kim *et al.*, 2002). We observed indeed a Dvl-mediated stimulation of LEF/TCF activity in MDCK cells, but two lines of evidence suggest that Dvl has LEF/TCF transcription-independent roles in MDCK morphogenesis: 1) Recombinant β -catenin molecules that are insensitive to GSK3 β -induced degradation induced LEF/TCF activation at levels comparable with those caused by Dvl. Yet stabilized β -catenin failed to mimic the phenotype of Dvl expression or GSK3 β inhibition. β -catenin Δ N90 (Figure 4) and β -catenin-S37A (our unpublished data) slightly delayed but did not inhibit tubule formation in collagen overlay and polarization in Ca²⁺-switch assays. This is in agreement with previous reports that MDCK cells expressing β -catenin Δ N90 are more motile and "fibroblastic" in nonconfluent monolayers but exhibit polarity comparable with control monolayers once they become confluent (Barth *et al.*, 1997). MDCK cells expressing Δ N90- β -catenin or a GSK3 β -phosphorylation defective full-length β -catenin mutant (β -catenin S4A) were competent to form cysts with central lumen in collagen matrices (Pollack *et al.*, 1997; Lyons *et al.*, 2004). β -catenin S4A cysts further responded to hepatocyte growth factor by undergoing branching tubulogenesis (Lyons *et al.*, 2004), although β -catenin Δ N90 cysts were unresponsive to hepatocyte growth factor (Pollack *et al.*, 1997). 2) In a scenario converse to that of β -catenin, we observed that a DEP-deficient mutant of Dvl was unable to stimulate LEF/TCF-mediated gene transcription, but it mimicked the phenotype described for wild-type Dvl.

Dvl Δ DEP has been shown to signal in a GSK3 β -dependent but transcription-independent pathway that leads to MT stabilization in neurons (Ciani *et al.*, 2004). Although LiCl, the most widely used GSK3 β inhibitor, phenocopied the effect of Dvl in our morphogenetic assays (our unpub-

lished data), no such effect was apparent when we inhibited GSK3 β function by more specific approaches. At 50 μ M, the specific GSK3 β inhibitor TDZD-8 (GSK3 β inhibitor I) caused a twofold stimulation of TCF/LEF activation in TOPFLASH assays, the maximum that we achieved with any GSK3 β inhibitor, but it failed to disturb MDCK polarization in the morphogenetic assays (our unpublished data). Likewise, siRNA-mediated down-regulation of GSK3 β by 80% was without any effect (our unpublished data). These findings suggest that GSK3 β does not function downstream of Dvl and Dvl Δ DEP in MDCK polarization and that LiCl might exert a GSK3 β -independent effect.

Apart from GSK3 β inhibition, a DEP-deficient Dvl construct has also been reported to activate rho in cultured mammalian cells. Although Habas *et al.* (2001) defined the Dvl PDZ and DEP domains as crucial for wnt-induced rhoA activation in human embryonic kidney 293 cells, Kishida *et al.* (2004) described a DEP-deficient Dvl mutant that was indistinguishable from the wild type in activating ROCK, a kinase downstream of rhoA in COS-7 cells. The DEP-deficient constructs used in the two studies differed in the region N-terminal of the DEP domain. Kishida's data indeed suggest a crucial role for a region between the PDZ and DEP domains in ROCK activation.

Rho and its downstream targets mDia and ROCK regulate cell-cell adhesion and cell shape changes in polarizing epithelial cells as well as tubulogenesis (Sahai and Marshall, 2002; Eisen *et al.*, 2006).

In the metanephric kidney, Dvl localizes to microfilaments and wnt signaling regulates the actin cytoskeleton (Torres and Nelson, 2000). These findings are consistent with a model, supported by our study, that some of the morphogenetic and transforming effects of wnt and Dvl might be mediated by changes in cell adhesion and actin organization. Ongoing studies in our laboratory are currently focused on testing whether the Par1b and Dvl pathways intersect in the regulation of actin dynamics via rhoGTPases.

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