Cdc42 and noncanonical Wnt signal transduction pathways cooperate to promote cell polarity

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Scratch-induced disruption of cultured monolayers induces polarity in front row cells that can be visualized by spatially localized polymerization of actin at the front of the cell and reorientation of the centrosome/Golgi to face the leading edge. We previously reported that centrosomal reorientation and microtubule polarization depend on a Cdc42-regulated signal transduction pathway involving activation of the Par6/aPKC complex followed by inhibition of GSK-3β and accumulation of the adenomatous polyposis coli (APC) protein at the plus ends of leading-edge microtubules. Using monolayers of primary rodent embryo fibroblasts, we show here that dishevelled (Dvl) and axin, two major components of the Wnt signaling pathway are required for centrosome reorientation and that Wnt5a is required for activation of this pathway. We conclude that disruption of cell–cell contacts leads to the activation of a noncanonical Wnt/dishevelled signal transduction pathway that cooperates with Cdc42/Par6/aPKC to promote polarized reorganization of the microtubule cytoskeleton.

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Abbreviations used in this paper: APC, adenomatous polyposis coli; aPKC, atypical protein kinase C; Dkk1, Dickkopf 1; Dvl, Dishevelled; MEF, mouse embryonic fibroblasts; REF, rat embryonic fibroblasts; sFRP1, soluble frizzled related protein 1.
accumulation of another tumor suppressor protein Dlg (Discs Large) in the plasma membrane at the leading edge. The subsequent association of microtubule-bound APC with membrane-bound Dlg is required for microtubule polarization and centrosome reorientation (Etienne-Manneville et al., 2005). It is likely that many other cellular activities are required for reorganization of the microtubule cytoskeleton; for example IQGAP, another Cdc42 effector, is required both for protrusion polarity as well APC and microtubule polarity (Watanabe et al., 2004) and the dynein/dynactin complex is required for centrosome reorientation and nDia and EB1, regulated by Rho, also contribute to APC localization and stabilization (Palazzo et al., 2001a; Wen et al., 2004).

In this report, we reexamined the significance of GSK-3 phosphorylation using fibroblasts derived from knock-in mice in which the phosphorylation sites of both GSK3α and β isoforms (Ser21 and Ser9, respectively) have been replaced with Ala (McManus et al., 2005). We find that GSK-3 phosphorylation is not required for Golgi/centrosome reorientation, but instead dishevelled (Dvl), axin, and Wnt ligands are required. It appears that a Cdc42/Par6/aPKC signaling pathway cooperates with a noncanonical Wnt signaling pathway to promote polarization of the microtubule cytoskeleton.

Results and discussion

GSK-3 phosphorylation is not required for centrosome/Golgi reorientation

We previously reported that localized inhibition of GSK-3β is required for centrosome/Golgi reorientation and that GSK-3β is phosphorylated downstream of Cdc42/Par6/PKCζ in migrating astrocytes and fibroblasts (Etienne-Manneville and Hall, 2003; Cau and Hall, 2005). To investigate whether phosphorylation is the mechanism of GSK-3 inhibition, we analyzed primary embryonic fibroblasts derived from double knock-in mice in which Ser21 of GSK-3α and Ser9 of GSK-3β have been replaced with nonphosphorylatable Ala residues (to be referred to as GSK-3SA) (McManus et al., 2005). After scratching a monolayer, GSK-3SA cells showed no defect in reorientation of the centrosome or the Golgi compared with littermate wild-type fibroblasts (GSK-3WT), both exhibiting ∼70% reorientation as early as 2 h after wounding (Fig. 1 A). The experiment is scored such that 33% corresponds to random orientation. Furthermore, small molecule inhibitors of GSK-3 blocked centrosome and Golgi reorientation in GSK-3SA and GSK-3WT cells to the same extent (Fig. 1 A). We conclude that although phosphorylation of GSK-3 occurs as a consequence of inducing directed cell migration, the spatially localized inhibition of GSK-3 occurs through a mechanism that does not involve GSK-3 phosphorylation.

Dishevelled and axin are required for centrosome/Golgi reorientation

An alternative mechanism of inhibiting GSK-3 activity is seen during Wnt signaling and involves protein–protein interactions mediated by dishevelled (Dvl). Wnt ligands induce the interaction of Dvl with the large scaffold protein axin, leading to dissociation of GSK-3 from a complex containing axin, APC, and β-catenin (Doble and Woodgett, 2003). To determine whether Dvl is required for centrosome reorientation, all three Dvl isoforms were depleted in rat embryo fibroblasts (REFs) using specific siRNA (Fig. 1 B). Depletion of single Dvl isoforms had varying partial effects, but depletion of all three Dvl isoforms completely inhibited centrosome and Golgi reorientation (Fig. 1 C; red line represents random orientation). This suggests that localized inhibition of GSK-3 is mediated by Dvl.

To analyze which domains of Dvl are required for reorientation, full-length Dvl2 or different Dvl domain constructs were

![Figure 1](https://example.com/figure1.png)

GSK-3 inhibition is mediated by dishevelled and not by phosphorylation. (A) Golgi reorientation toward the scratch was quantified in GSK-3WT and GSK-3SA MEFs. Cells were pretreated for 1 h with indicated inhibitors, or 0.1% DMSO as control: 20 μM SB216763 or 5 μM CHIR99021 [both are GSK-3 inhibitors]. *, P < 0.001; **, P < 0.01 in t test when compared with DMSO control. (B) Dvl1, 2, and 3 are efficiently depleted using specific siRNAs, also in combination (depletion >95%), compared with nonspecific control siRNA oligo. aPKC serves as loading control. Arrows indicate position of Dvl protein. (C) Golgi and centrosome reorientation were measured in cells depleted of single Dvl isoforms or their combinations. Red bar marks basal level expected from random Golgi/centrosome reorientation (33%). *, P < 0.01; **, P > 0.05, in t test when compared with control.
microinjected into the nuclei of leading edge cells immediately after scratching a REF monolayer (Fig. 2, A and B). Cells expressing full-length DvI2 exhibited complete loss of reorientation (Fig. 2 B). Overexpression of DvI also blocked centrosome reorientation in GSK-3β knock-in cells (unpublished data). This suggests that overexpression of DvI interferes with the polarized inhibition of GSK-3, as has been observed in other situations (Itoh and Sokol, 1997; Boutros et al., 1998; Modzik, 2002). Constructs containing the DIX domain (DIX and DIX+PDZ) were also able to block reorientation, while the PDZ alone, DEP domain, or ∆DIX had no effect on reorientation (Fig. 2 B), suggesting that DvI regulates reorientation through its DIX domain.

The DIX domain interacts with DvI itself and with axin (Kishida et al., 1999). To examine whether axin is required for reorientation, siRNA was used (Fig. 2, C and D). Interestingly, axin depletion blocked reorientation to a similar degree as DvI2 or DvI3 depletion (Fig. 2 D). Moreover, when axin was depleted together with DvI2 and DvI3, reorientation was completely inhibited, suggesting that DvI2 and DvI3 cooperate with axin, likely through a direct interaction. Axin overexpression after microinjection of an expression construct also blocked reorientation, whereas expression of axin lacking the DIX domain did not (Fig. 2 E), emphasizing the important role for the DIX domain in this process.

**Wnt ligands are required for reorientation upstream of dishevelled**

Because DvI and axin are key components of Wnt signaling pathways and are also required for centrosome/Golgi reorientation, we tested whether Wnt ligands themselves might be involved. Wnt signaling was blocked using recombinant soluble Frizzled-related protein 1 (sFRP1), a naturally occurring antagonist of the Wnt pathway, which acts by binding to Wnt ligands and sequestering them away from frizzled receptors (Jones and Jomary, 2002). sFRP1 treatment led to substantial inhibition of centrosome/Golgi reorientation, suggesting that Wnts are involved in reorientation (Fig. 3 A). In contrast, recombinant Dickkopf1 (Dkk1) protein, another antagonist that specifically inhibits a subset of Wnt ligands that are required only for the canonical Wnt pathway, did not significantly block reorientation, implying that one or more Wnts belonging to the noncanonical family are responsible (Kawano and Kypri, 2003). Importantly, both antagonists blocked β-catenin stabilization in L-cells treated with Wnt3a, confirming their activity as negative regulators of Wnt signaling (unpublished data).

DvI activity has been reported to be controlled by multiple phosphorylation events (Willert et al., 1997; McKay et al., 2001; Sun et al., 2001), which can be observed as a series of band shifts on Western blots (Gonzalez-Sancho et al., 2004; Endo et al., 2005). To examine whether the block in centrosome reorientation by sFRP1 impacts DvI phosphorylation, we examined DvI2 and DvI3 mobility on 7.5% polyacrylamide-SDS gels (Fig. 3 B). DvI2 and DvI3 appear as multiple bands even in unscratched, confluent REF monolayers, suggesting some constitutive phosphorylation (Fig. 3 B, top panel lanes 1 and 9). Addition of sFRP-1, but not Dkk-1 caused a significant increase in gel mobility (Fig. 3 B, lanes 5 and 13, respectively), suggesting that DvI is phosphorylated in monolayers and that this is...
dependent on a constitutive, noncanonical-like Wnt activity. Scratching the monolayer resulted in no major changes in the mobility of Dvl.

**Wnt5a is a potential ligand for controlling microtubule polarity**

Recent reports describe Wnt5a as a regulator of noncanonical Wnt signaling, specifically during events requiring cell migration in vertebrates (Myers et al., 2002; De Calisto et al., 2005; Kim et al., 2005; Matsui et al., 2005; Okuse et al., 2005; Qiang et al., 2005; Nishita et al., 2006). This prompted us to examine a possible role for Wnt5a in polarized cell migration. Wnt5a protein could be detected by Western blot, though its levels remained constant throughout a 2-h time course after scratching, confirming that primary fibroblasts constitutively produce Wnt5a (Fig. 3 C). To address the role of Wnt5a in centrosome/Golgi reorientation, Wnt5a was depleted using two different specific siRNA oligonucleotides (Fig. 3 D). Both siRNA oligonucleotides led to substantial inhibition of reorientation, suggesting that Wnt5a is likely to be the major Wnt ligand regulating polarity in these cells (Fig. 3 E). siRNA-mediated depletion of two other Wnt ligands, Wnt1 and Wnt3a, did not block reorientation (unpublished data). Furthermore, Dvl mobility on gels was significantly increased by both Wnt5a siRNA oligos, suggesting that Dvl phosphorylation is dependent on Wnt5a (Fig. 3 F). Although we cannot exclude the possibility that other Wnts are expressed and involved, these data support an important role for Wnt5a in polarization of the centrosome/Golgi.

**Wnt and dishevelled signaling are required for APC recruitment to microtubule plus tips**

A key step in the pathway controlling centrosome/Golgi reorientation is APC recruitment to microtubule plus ends (Etienne-Manneville and Hall, 2003; Etienne-Manneville et al., 2005). In control scratched monolayers, APC is localized along microtubules but is enriched at the leading edge (Fig. 4 A). In Dvl-depleted cells, APC is mislocalized; it is found along all microtubules at the back as well as the front of the cell and is enriched in the perinuclear region (Fig. 4 B). APC localization was also greatly affected by axin or Wnt5a depletion (Fig. 4, C and D, respectively), showing loss of microtubule tip accumulation and concentration of APC in the perinuclear region. APC localization in cells treated with sFRP1 was similar to that of cells treated with Wnt5a siRNA (unpublished data). Quantifying the effects of siRNA on APC localization demonstrates that Dvl, axin, and Wnt5a are all required for proper APC polarization (Fig. 4 E).
aPKC interacts with Dvl2 after scratching

Dvl can be regulated by protein–protein interactions, leading to changes in its subcellular localization and subsequent complex formation (Cliffe et al., 2003; Ciani et al., 2004; Dollar et al., 2005; Park et al., 2005). Because we have previously shown that the Par6/aPKC/Cdc42 complex is also required for polarized APC accumulation, we looked for a biochemical interaction between these two signaling pathways. Dvl2 was immunoprecipitated from cells pretreated with an aPKC inhibitor (Go6983), a Wnt inhibitor (sFRP1), or a Cdc42 inhibitor (Toxin B10463). In untreated cells, aPKC could be found in a complex with Dvl2 and Dvl3, and this was increased upon scratching (Fig. 5 A). Although the increase is modest, scratching induces localized changes in signaling activities at the front of the cell and this is very different, for example, from adding a soluble agonist. When Wnt signaling or Cdc42 activity was blocked, the induced, but not the basal level of this interaction was lost, suggesting both Wnt and Cdc42 activities are required to promote a Dvl2/aPKC interaction after scratching (Fig. 5, B and C). In contrast, aPKC inhibitors did not block this interaction, suggesting aPKC activity was not required for Dvl2/aPKC complex formation.

In conclusion, whereas Dvl, axin, APC, and GSK-3 participate in canonical Wnt signaling to promote a transcriptional response, the Wnt5a/dishvelled effects described here represent a noncanonical pathway that is independent of transcription and involves polarization of microtubules. Although Wnt5a is constitutively expressed in these cells, the scratch-induced generation of a new leading edge leads to spatially localized activation of the Cdc42/Par6/aPKC complex and these two pathways are both required to control polarity. The regulation of polarity by Wnt5a could therefore occur by (a) its polarized secretion, (b) polarized activation of its receptor, or (c) polarized activation of the downstream signal transduction pathway (Strutt, 2002, 2003; Wu et al., 2004). The biochemical link between Cdc42/Par6/aPKC and Wnt5a/Dvl is not clear, though we have identified an interaction between aPKC and Dvl2, which is increased soon after scratching. The results described here provide new insights into the role of noncanonical Wnt pathways in establishing microtubule polarity and identify a potential link between Cdc42 and Wnt signaling pathways, whereby polarization of Wnt signaling can occur in a Cdc42-dependent manner, in response to an external cue.

Materials and methods

Reagents and antibodies

The following antibodies were used: mouse anti-Dvl1, rabbit anti-Dvl2 (used for Western blot), mouse anti-Dvl3, rabbit anti-aPKC, mouse anti-axin (Santa Cruz Biotechnology, Inc.), rabbit anti-axin (Zymed Laboratories), goat anti-Wnt5a (R&D Systems), mouse anti-p115 (Golgi marker), mouse anti-tubulin (used for IF), mouse anti-myc (Cancer Research UK, London), rabbit anti-pericentrin ( Covance), rat anti-a-tubulin (used for Western blot) (Harlan), and rabbit anti-APC (a gift from...
Figure 5. Dvl2 and aPKC form a complex after scratching in a Wnt- and Cdc42-dependent manner. (A) Endogenous Dvl2 was immunoprecipitated using monoclonal antibodies against Dvl2 or Dvl3 from cell extracts 30 min after scratching. Top panel shows communoprecipitated aPKC and immunoprecipitated Dvl2. Western blotting was done using a rabbit antibody against Dvl2, and middle panel shows the communoprecipitated aPKC and immunoprecipitated Dvl3. aPKC levels in 10% of total input extract were used as loading control (bottom panel). Control immunoprecipitation (right panel) using a nonspecific antibody (mouse anti-Myc) demonstrates the specificity of aPKC immunoprecipitations. (B) Endogenous Dvl2 was immunoprecipitated from extracts of cells that had been pretreated for 1 h with 8 μM Go6983 (aPKC inhibitor), 5 μg/ml sFRP1 (Wnt inhibitor), or 5 ng/ml Toxin B10463 (Rho GTPase inhibitor) and then scratched and left for 30 min. Top two panels show communoprecipitated aPKC and immunoprecipitated Dvl2. aPKC levels in 10% of total input extract were used as loading control. (C) Quantification of fold increase in Dvl2-aPKC interaction after scratching cell monolayer is shown. aPKC levels were normalized to Dvl2 levels (i.e., the sum of the two migrating species). Average of three independent experiments is shown. Red bars mark basal (un-induced) levels.

Inke Nathke, University of Dundee, UK). Secondary antibodies coupled to HRP were from Jackson Immunoresearch Laboratories. Secondary antibodies used for immunofluorescence and fluorescent phalloidin were from Molecular Probes. The following reagents were used: recombinant human sFRP-1 and recombinant human Dkk-1 (R&D Systems), Go6983, Toxin B10463 (Calbiochem), SB216763 (Tocris), and CHIR99021 (a gift from Philip Cohen, University of Dundee, UK).

cDNA constructs and cloning procedures

Full-length cDNA of mouse Dvl2 was a gift from Trevor Dale (Institute of Cancer Research, UK). Deletion mutants of Dvl2 were made using PCR amplification of fragments of interest and subcloning into pRK5myc. All sequences were confirmed by sequencing (MWG Biotech). RFP-F, a farne-sylated form of RFP that is targeted to the membrane, was described previously (Cau and Hall, 2005). Axin constructs were a gift from Robert Kypta (Hammersmith Hospital, UK).

Cell culture and microinjection

Primary rat embryonic fibroblasts (REF) were prepared as described previously (Cau and Hall, 2005). Primary mouse embryonic fibroblasts (MEF) were provided by McManus et al. (2005). Only cells from passage 2–3 were used in all experiments. Both cell types were maintained in DME supplemented with 10% fetal calf serum, streptomycin, and penicillin at 37°C. MEFs were maintained in 10% CO2 and MEF in 5% CO2. Microinjections were performed as previously described (Cau and Hall, 2005). In brief, confluent cell monolayers grown on glass coverslips were scratched with a P2 tip. 30 min later, cells along the wound edge were microinjected with 0.4 μg/μl cDNA of interest plus 0.15 μg/μl pRFP-F, which served as an injection tracer and membrane marker. Cells were fixed 4.5 h later.

Quantification of Golgi, centrosome, and APC polarization

Polarization was determined 4.5 h or 2 h after scratching of REF or MEF monolayers, respectively (Cau and Hall, 2005). Golgi was detected using antibodies against p115 and centrosome was detected using antibodies against pericentrin. First row cells showing the Golgi/centrosome located in front of the nucleus and in the 120° sector facing the wound, were counted as oriented (Etienne-Manneville and Hall, 2001; Cau and Hall, 2005). For APC polarization, cells exhibiting front accumulation of APC were counted as polarized. Cells exhibiting uniform distribution or perinuclear staining were counted as nonpolarized. For drug-treated or siRNA-treated cells at least 100 cells were counted per coverslip. For microinjected coverslips at least 50 cells were counted per coverslip. Each data point represents at least three independent experiments. Error bars represent SD and statistical tests were performed using a two-tailed t test with equal variances.

RNAi experiments and nucleofections

Unless otherwise specified, all double-stranded predesigned HPLC-grade siRNA oligos were obtained from MWG Biotech. siRNA treatment was performed as previously described (Etienne-Manneville and Hall, 2001; Cau and Hall, 2005). In brief, a total of 600 ng of siRNA oligos were introduced into 1.5 × 105 cells by nucleofection (Amaxa) using solution V, program G09. Cells were replated at a density of 5 × 104/cm2 and assayed 72 h later. Efficient depletion was observed 2–3 d after nucleofection, and was quantified by Western blot. The following siRNA oligos were used: rat Dvl1 (Ambion) GAGGAGAAGUCUUGACGACUtr, rat Dvl2 GUACGCUUUUGAGACGUCUCAc, rat Dvl3 GAGAGAAGACUCAGc, rat axin GUAUCGUUGUGCCUCUAttr, rat Wnt5a (#1) UAAACCGUAUAUCCACAUAtt, rat Wnt5a (#2) GCCAGUGGAAACACUUUctt, non-specific control AGGUAGUUGUAUCGCUCUGt.
Confluent cells were taken with a MRC1024 (Bio-Rad Laboratories) confocal OPTIPLATT® [Nikon] microscope using a 60× objective [NA 1.4] and a Kr/Ar laser. Immunofluorescent images were taken with Axioplan microscope using a 63× oil immersion objective [NA 1.4]. Images were acquired with an ORCA-ER [Hamamatsu] camera using Openlab software [Improvision]. Images were analyzed and processed for presentation using brightness and contrast adjustments [same settings for all images] and Adobe Photoshop CS.

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