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Article

Par3 Controls Epithelial Spindle Orientation by aPKC-Mediated Phosphorylation of Apical Pins

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Summary

Background: Formation of epithelial sheets requires that cell division occurs in the plane of the sheet. During mitosis, spindle poles align so the astral microtubules contact the lateral cortex. Confinement of the mammalian Pins protein to the lateral cortex is essential for this process. Defects in signaling through Cdc42 and atypical protein kinase C (aPKC) also cause spindle misorientation. When epithelial cysts are grown in 3D cultures, misorientation creates multiple lumens.

Results: We now show that silencing of the polarity protein Par3 causes spindle misorientation in Madin-Darby canine kidney cell cysts. Silencing of Par3 also disrupts aPKC association with the apical cortex, but expression of an apically tethered aPKC rescues normal lumen formation. During mitosis, Pins is mislocalized to the apical surface in the absence of Par3 or by inhibition of aPKC. Active aPKC increases Pins phosphorylation on Ser401, which recruits 14-3-3 protein. 14-3-3 binding inhibits association of Pins with Gαi, through which Pins attaches to the cortex. A Pins S401A mutant mislocalizes over the cell cortex and causes spindle orientation and lumen defects.

Conclusions: The Par3 and aPKC polarity proteins ensure correct spindle pole orientation during epithelial cell division by excluding Pins from the apical cortex. Apical aPKC phosphorylates Pins, which results in the recruitment of 14-3-3 and inhibition of binding to Gαi, so the Pins falls off the cortex. In the absence of a functional exclusion mechanism, astral microtubules can associate with Pins over the entire epithelial cortex, resulting in randomized spindle pole orientation.

Introduction

Orientation of the mitotic spindle is essential for asymmetric stem cell divisions and for tissue morphogenesis [1]. In the *Drosophila* neuroblast, the polarity proteins Par3, Par6, and atypical protein kinase C (aPKC) form a complex that is organized into a crescent at the apical cortex [2, 3]. Par3 binds to an adaptor protein called Inscuteable, which in turn recruits Partner of Inscuteable (Pins) to the apical crescent. A second pathway involving the heterotrimeric G protein Gαi, Discs large (Dlg), and microtubules also helps ensure localized enrichment of Pins [4]. Pins is believed to attach astral microtubules to the cortex, ensuring correct spindle orientation so that the apical daughter retains the Par and Pins proteins while cell fate determinants are segregated into the basal daughter. A related process controls spindle orientation in the *C. elegans* zygote [5, 6], but the mechanisms in other cell types are less well understood.

Epithelial monolayers are a basic unit of organization in many tissues and emerge through a combination of intercellular adhesion and oriented cell division [7, 8]. Epithelial cells possess an apical-basal polarity, and intercellular adhesion occurs through the lateral membranes. Extension of epithelial sheets requires that cell division occurs in the plane of the sheet. Several polarity proteins have been implicated recently in spindle pole orientation during epithelial cell division, including Cdc42 [9], the Cdc42-specific exchange factors Tuba [10] and Intersectin-2 [11], aPKC [10], and the mammalian Pins protein, also called LGN [12]. Cdc42-GTP can bind to the Par6/aPKC complex and activate aPKC [13]. Downstream of aPKC, Pins/LGN has to be excluded from the apical cortex so as to ensure the correct orientation of the mitotic spindle. Either the inhibition of aPKC or the forced tethering of Pins to the apical surface will severely disrupt spindle orientation. However, the underlying mechanism that controls Pins exclusion from the apical cortex remains unclear.

When Madin-Darby canine kidney (MDCK) or Caco-2 epithelial cells are grown in Matrigel 3D cultures, they form highly polarized cysts in which the apical surface faces a single central lumen [8, 14]. This system has proven to be a valuable in vitro model of epithelial morphogenesis and recapitulates many of the processes that occur during the formation of ducts. Mitosis occurs in the plane of the cyst surface, such that the cysts maintain a single layer of cells as they enlarge. Inhibition of aPKC or the loss of Cdc42 disrupts spindle pole orientation, which causes the formation of multiple lumens [9–11].

Using this system, we show that silencing of Par3 expression in MDCK cells also disrupts spindle pole orientation, through the mislocalization of aPKC away from the apical surface. Atypical PKC can phosphorylate Pins on Ser401, which enhances binding of 14-3-3. In most cells, Pins is recruited to the cell cortex through association not with Inscuteable but with the heterotrimeric G protein Gαi, to which it binds via GoLoco domains in its C-terminal region [15–17]. We find that 14-3-3 binding to Pins inhibits this association, which will result in the release of Pins from the cortex. Thus, aPKC-mediated exclusion of Pins from the apical cortex ensures that astral microtubules will not attach to the apical surface and that mitosis occurs only in the plane of the epithelial sheet.

Results

Silencing of Par3 Causes a Spindle Orientation Defect

The polarity protein Par3 was silenced in MDCK cells by expression of a short hairpin RNA (shRNA) from a plasmid or

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lentivirus. As reported previously [18], depletion of Par3 resulted in a robust defect in lumen formation when the cells were grown as cysts in Matrigel cultures, (Figures 1A and 1B). Two different shRNAs gave similar phenotypes and efficiently silenced Par3 expression (Figure 1C). Importantly, however, the cells still retained normal apical-basal polarity as assessed by the apical markers podocalyxin/gp135 and actin, the tight junction (TJ) marker ZO-1, and the lateral markers E-cadherin, α-catenin, and β-catenin (Figures 1A and 1D).

In 2D monolayer cultures, loss of Par3 causes a delay in TJ assembly, through the inappropriate activation of the Rac GTPase and LIMK2 [19, 20]. The C-terminal region of Par3 can bind directly to the Rac exchange factor Tiam1, thereby restricting its distribution in the cell and its activation of Rac. This restriction is lost upon knockdown of Par3. To test whether a similar signaling pathway controls lumen formation in MDCK cell cysts, we coexpressed the Par3 shRNA with a dominant-negative Rac(N17) mutant and shRNA against Tiam1, both of which we had shown previously to rescue TJ assembly in the absence of Par3 [20]. However, neither approach restored single lumen formation (Figures 1E and 1F; see also Figures S1A and S1B available online). We therefore conclude that Par3 does not act through the Tiam1/Rac pathway in the organization of lumens.

The multilumen phenotype can occur if epithelial cells undergo mitosis in the wrong plane [9]. Therefore, we next tested whether spindle orientation might be defective when cells are depleted of Par3. It is difficult to assess spindle angles when cysts have multiple lumens. However, when cells are immediately plated into Matrigel after transduction with a Par3-shRNA lentivirus, cysts grow and begin to form lumens before depletion of the Par3 protein has occurred, and at 3 days posttransduction, most of these cysts still retain a single lumen, so spindle orientation of mitotic cells can be easily assessed by reference to the apical surface of the cyst (Figure 1G). Using this method, we found that silencing of Par3 expression causes a strong defect in spindle orientation, with a large fraction of the cells dividing in a direction perpendicular to the plane of the cyst surface (Figures 1H and 1I). Based on these data, we suggest that multiple lumens arise because of the spindle orientation defect caused by loss of Par3.

Spindle Misorientation Is Caused by Loss of Apical aPKC

In mammary gland luminal epithelial cells, the depletion of Par3 causes a loss of aPKC from the apical surface [21]. A similar phenotype was observed in MDCK cysts, in which lentivirally driven knockdown of Par3 eliminated aPKC from the apical surface, but with no change in total level of the kinase (Figures 2A–2C). We next asked whether this mislocalization was causally related to a spindle orientation defect. For this purpose, we constructed a fusion of full-length, constitutively active aPKC to the PDZ domains of NHERF2 (Figure 2D; Figure S1C). NHERF1 and 2 are regulators of the Na/H exchanger and localize to the apical surface through a PDZ domain-mediated interaction with the C terminus of podocalyxin/gp135 [22, 23]. Because apical-basal polarity of the MDCK cells is not lost when Par3 is depleted (Figure 1), we reasoned that expression of this fusion protein would tether aPKC to the apical cortex in a Par3-independent manner. When the construct was coexpressed with the Par3 shRNA, it was enriched at the apical cortex (Figure 2E) and the majority of cysts developed single lumens (Figures 2E and 2F). In addition, spindle pole orientation was normalized (Figure 2E). Expression of a kinase-dead aPKC increased the number of defective cysts, whereas an untethered active aPKC had no effect, confirming that apical localization is important to maintain normal spindle orientation. We conclude that active aPKC at the apical cortex is required to prevent mitosis from occurring in the wrong plane.

Pins Is Excluded from the Apical Cortex by aPKC-Dependent Phosphorylation

The mammalian Pins protein (Pins or LGN) plays essential roles in spindle orientation during mitosis of both stem cells and epithelial cells [1, 12]. Pins/LGN is confined to the lateral cortex in dividing MDCK cells, and the expression of an apically tethered Pins causes a profound orientation defect [12]. Moreover, the apical exclusion of endogenous Pins is lost when aPKC is inhibited. Together, these data suggested that Pins might be phosphorylated by aPKC. Consistent with this idea, depletion of Par3 results in the mislocalization of Pins in mitotic MDCK cells, such that it is no longer excluded from the apical surface (Figures 3A and 3B). To test whether Pins can be phosphorylated by aPKC, we expressed Myc-tagged Pins in 293T cells together with a membrane-tethered aPKC (myr-aPKC) or a T410E constitutively active mutant (CA-aPKC), or with added myristoylated pseudosubstrate to inhibit endogenous aPKC. The cells were incubated in medium containing [^{32}P]phosphate, and the Pins was purified from lysates over Myc antibody-conjugated agarose beads. The incorporation of ^{32}P into Pins was increased in the presence of activated aPKC as compared to wild-type aPKC and was reduced by the pseudosubstrate inhibitor (Figure 3C).

Ser401-Phosphorylated Pins Binds 14-3-3

A subset of sites phosphorylated by aPKC are recognized by the 14-3-3 family of phospho-Ser binding proteins [24, 25]. We found that HA-tagged 14-3-3 coprecipitated with myc-Pins but that this interaction was blocked by the aPKC pseudosubstrate inhibitor (Figure 3D). Robust binding was also observed when a constitutively active aPKC was expressed with the Pins and 14-3-3; importantly, aPKC itself did not bind to Pins (Figure S3A).

The N-terminal TPR repeat domain of Pins associates in an intramolecular interaction with the C-terminal domain, which contains GoLoco motifs [26], and can switch between open and closed conformational states [16, 17]. NuMA interacts with the TPR repeats while Gαi-GDP binds the GoLoco motifs. The closed state binds only inefficiently to Gαi subunits; NuMa stabilizes Pins in its open conformation and enhances Gαi binding. Deletion of the N-terminal region also enhances Gαi binding [17]. To identify the region of Pins that interacts with 14-3-3, we expressed the isolated N terminus, the Pins ΔN fragment, or full-length Pins together with HA-tagged 14-3-3 and active aPKC. As shown in Figure 3E, although Pins ΔN was expressed less efficiently than the other two constructs, it bound 14-3-3 much more robustly than either of them (Figure 3D). (The weak but detectable binding of the N-terminal fragment might arise from its association with endogenous Pins, which would be forced into the open conformation.) These data show that 14-3-3 recognizes phosphorylated Pins and binds to the linker and/or C-terminal domain. Moreover, this interaction seems to be suppressed by the N-terminal domain.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis revealed multiple phosphorylations of Ser and Thr residues in S-tagged full-length Pins coexpressed

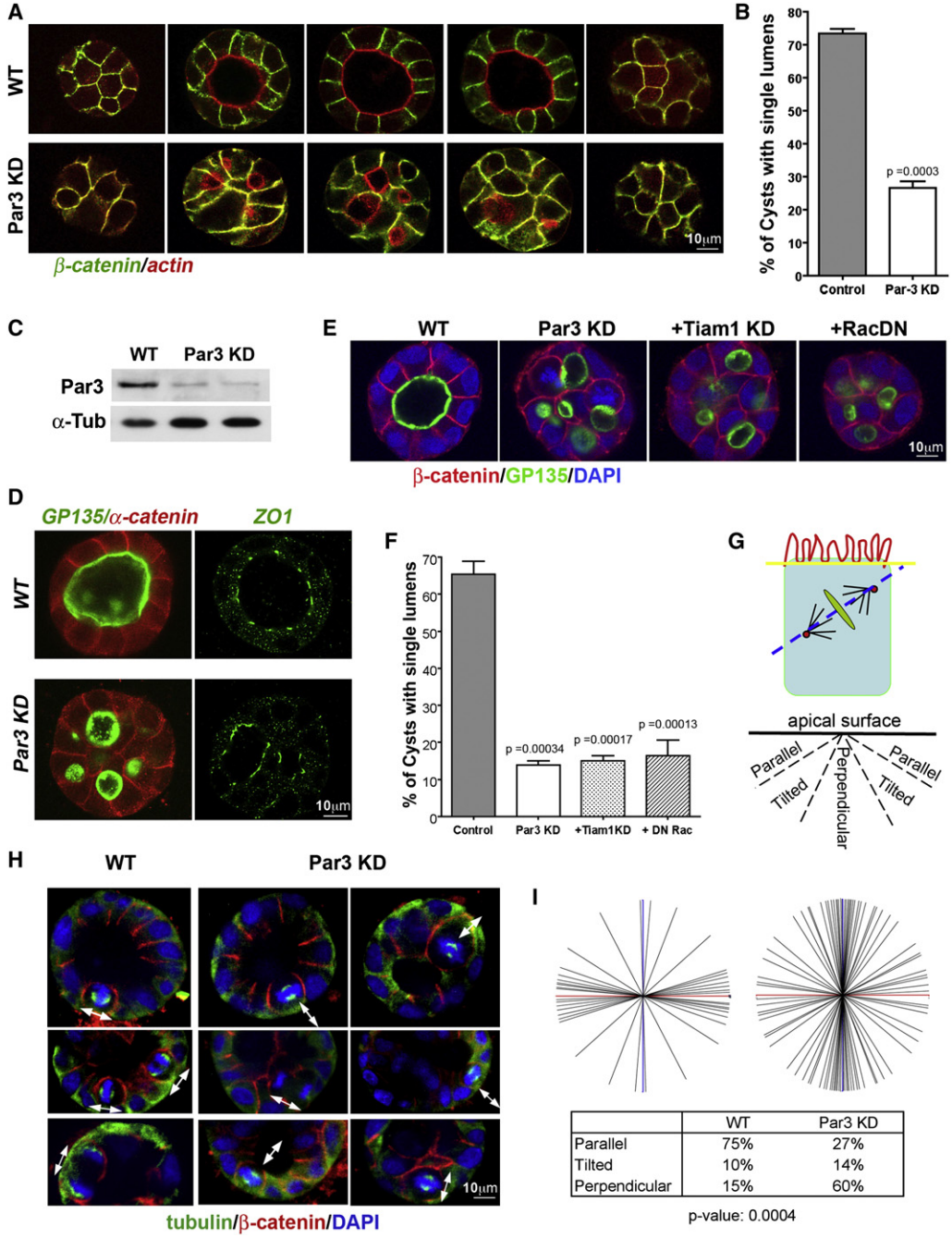

Figure 1. Loss of Par3 Causes Defects in Lumen Formation and Spindle Orientation through a Rac/Tiam1-Independent Mechanism

(A and B) Silencing of Par3 expression produces cysts that retain apical/basal polarity but form multiple lumens.

(A) Z sections through cysts stained for β-catenin (green) and actin (red).

(B) Cysts were scored for lumen formation and compared by unpaired t test (n > 100 cysts per condition; error bars are ± one standard deviation [SD]).

(C) Knockdown efficiency of the Par3 shRNAs. Two plasmid shRNAs against different canine Par3 DNA sequences were used [20] and produced similar levels of knockdown. The phenotypes were indistinguishable.

(D) Apical and tight junction markers are not disrupted by silencing of Par3. Cysts expressing control or Par3 shRNAs were stained for α-catenin (red) as a lateral marker and podocalyxin/gp135 (green) as an apical marker, or ZO-1 (green) to mark the tight junctions.

(E and F) The lumen defect caused by silencing of Par3 is not reversed by cosilencing of Tiam1 or coexpression of a dominant-negative mutant of Rac [20]. Representative images (E) and quantification (F) of lumen formation are shown (n > 50 cysts per condition; error bars are ± one SD).

(G–I) Loss of Par3 disrupts mitotic orientation.

(G) Angles of mitosis were measured relative to the plane of the apical surface.

(H and I) Representative images (H) (green, tubulin; red, β-catenin; blue, DAPI) and quantification (I) of angles of mitosis. Data were analyzed by Mann-Whitney test. Means were significantly different, p = 0.0004 (n = 66).

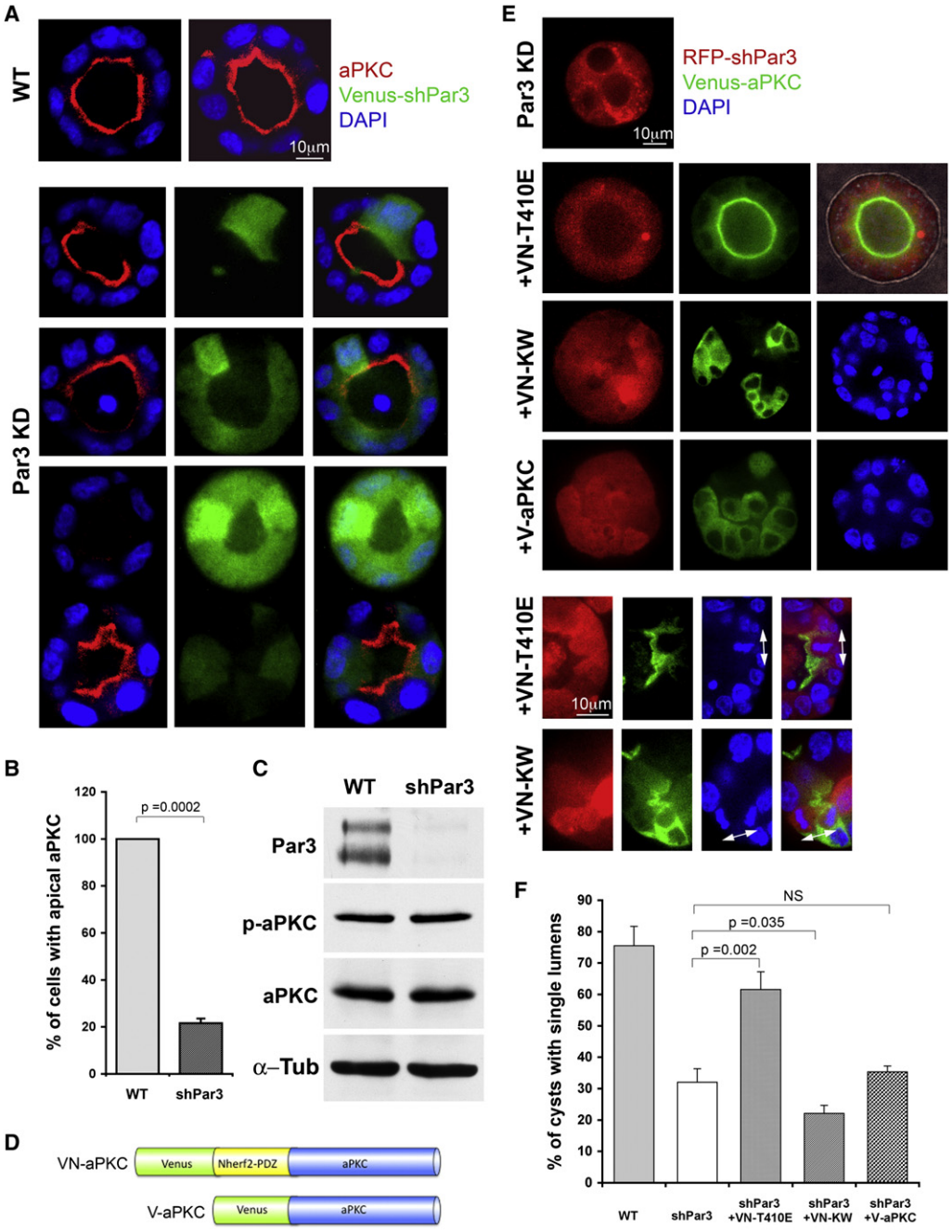

Figure 2. Lumen Organization Requires Apical aPKC, which Is Recruited by Par3

(A–C) Loss of Par3 results in the disappearance of aPKC from the apical cortex (A), but with no change in the overall level of aPKC expression (C). Cysts were scored for cells with apical aPKC localization and compared by unpaired t test (B) (n = 60; error bars are ± one SD).

(D–F) Apically tethered aPKC rescues normal cyst morphogenesis in the absence of Par3 (E). A schematic of the aPKC fusion proteins is shown in (D). The NHERF2-PDZ domains bind podocalyxin, thereby recruiting the fusion protein to the apical cortex. An activated aPKC fusion (VN-T410E) rescues normal lumen formation, whereas the kinase-dead mutant (VN-KW) and wild-type untethered aPKC (V-aPKC) do not (F). Error bars are ± one SD.

with activated aPKC (data not shown). All phosphorylated residues were situated in the linker region or C-terminal domain. Further analysis using the Pins ΔN fragment identified the same sites (Figures S1D and S2; Table S1). Notably, S401 was stoichiometrically phosphorylated (~98%; Figure S2). Seven of the mapped phosphorylation sites (S401, S434, S471, S534, S560, S631, and S670) were mutated to Ala residues and tested for their effects on 14-3-3 binding. None of the mutations had any effect except for S401A, which almost completely abolished 14-3-3 binding (Figure 4A). This mutant also showed no increase in ^{32}P incorporation when coexpressed with activated aPKC (Figure 4B). As expected, the phosphomimetic mutant Pins S401E also did not bind to 14-3-3 (Figure S3C). We therefore conclude that aPKC phosphorylates (either directly or indirectly) S401 in the linker region of Pins, which permits the association of Pins with 14-3-3.

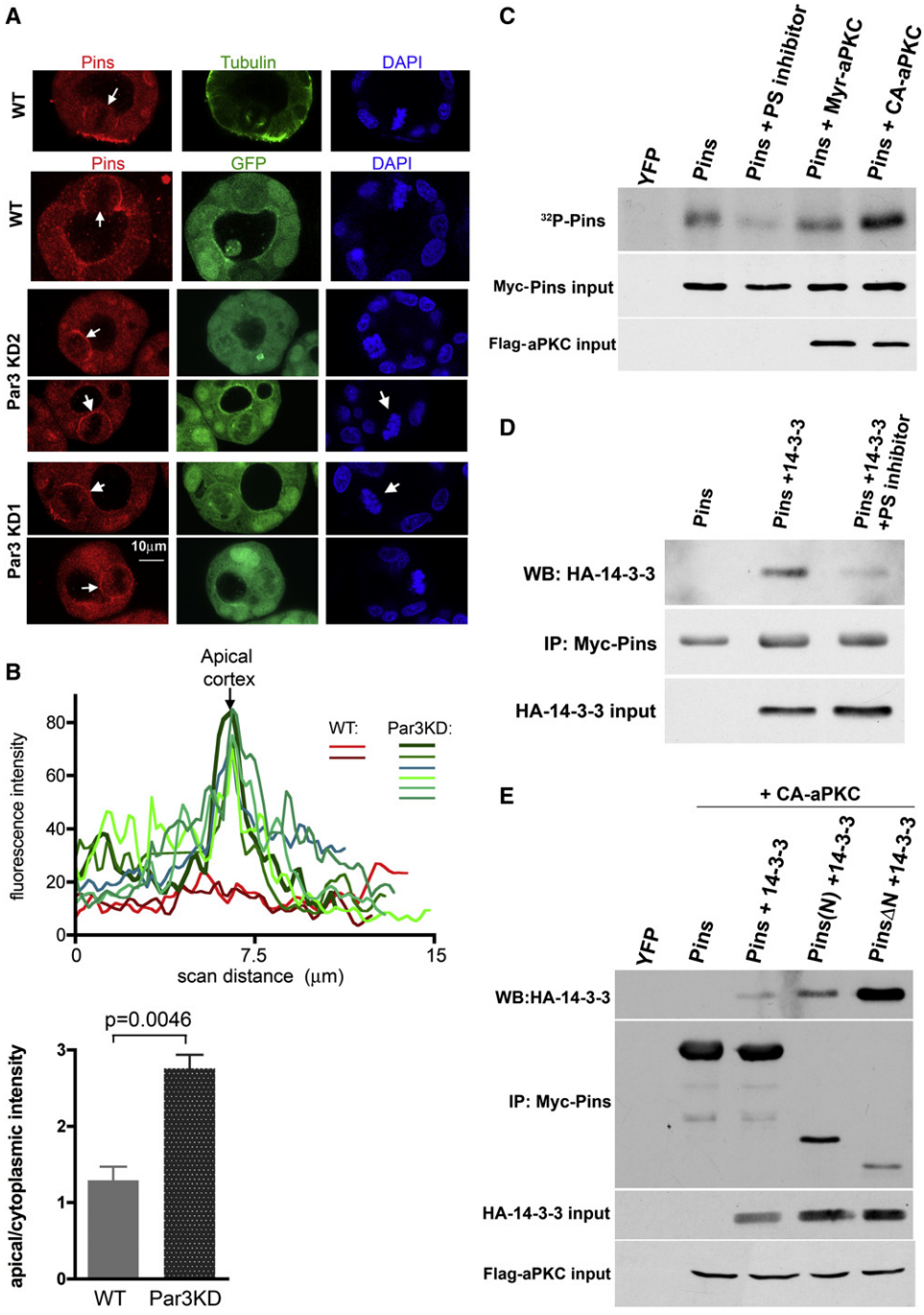

Figure 3. Loss of Par3 Causes Mislocalization of Pins, which Is Phosphorylated by aPKC and Binds 14-3-3

(A) Endogenous Pins localization in mitotic MDCK cells. Cells were untransfected (top panels) or transfected with control or Par3 shRNAs (other panels). Cysts were fixed and stained for Pins, DNA, and tubulin (top panels) or GFP (other panels). White arrows mark the enrichment of Pins on the cortex of mitotic cells.

(B) Top: line scans across the apical cortex of mitotic cells stained for endogenous Pins. Bottom: histogram showing mean ratios of apical to cytoplasmic intensity of Pins staining. p value was calculated by unpaired, two-tailed t test; error bars are ± one SD.

(C) Pins is phosphorylated by aPKC. Myc-tagged Pins was immunoprecipitated from 293T cells incubated with [^{32}P]phosphate. Coexpression of activated aPKC increased Pins phosphorylation, and a pseudosubstrate (PS) aPKC inhibitor peptide reduced Pins phosphorylation.

(D) Active aPKC promotes binding of 14-3-3 to Pins. Myc-Pins was immunoprecipitated from cells expressing HA-14-3-3 with or without activated aPKC, or plus pseudosubstrate inhibitor.

(E) A fragment of Pins lacking the N-terminal TPR repeats binds strongly to 14-3-3. Full-length Pins, the isolated N-terminal region (Pins(N)), or the linker plus GoLoco motifs (Pins ΔN) was expressed with HA-14-3-3 plus activated aPKC, immunoprecipitated, and blotted for the HA tag. (The Pins(N) might associate with more 14-3-3 than the full-length protein by associating with the C terminus of endogenous Pins and forcing it into the open conformation.)

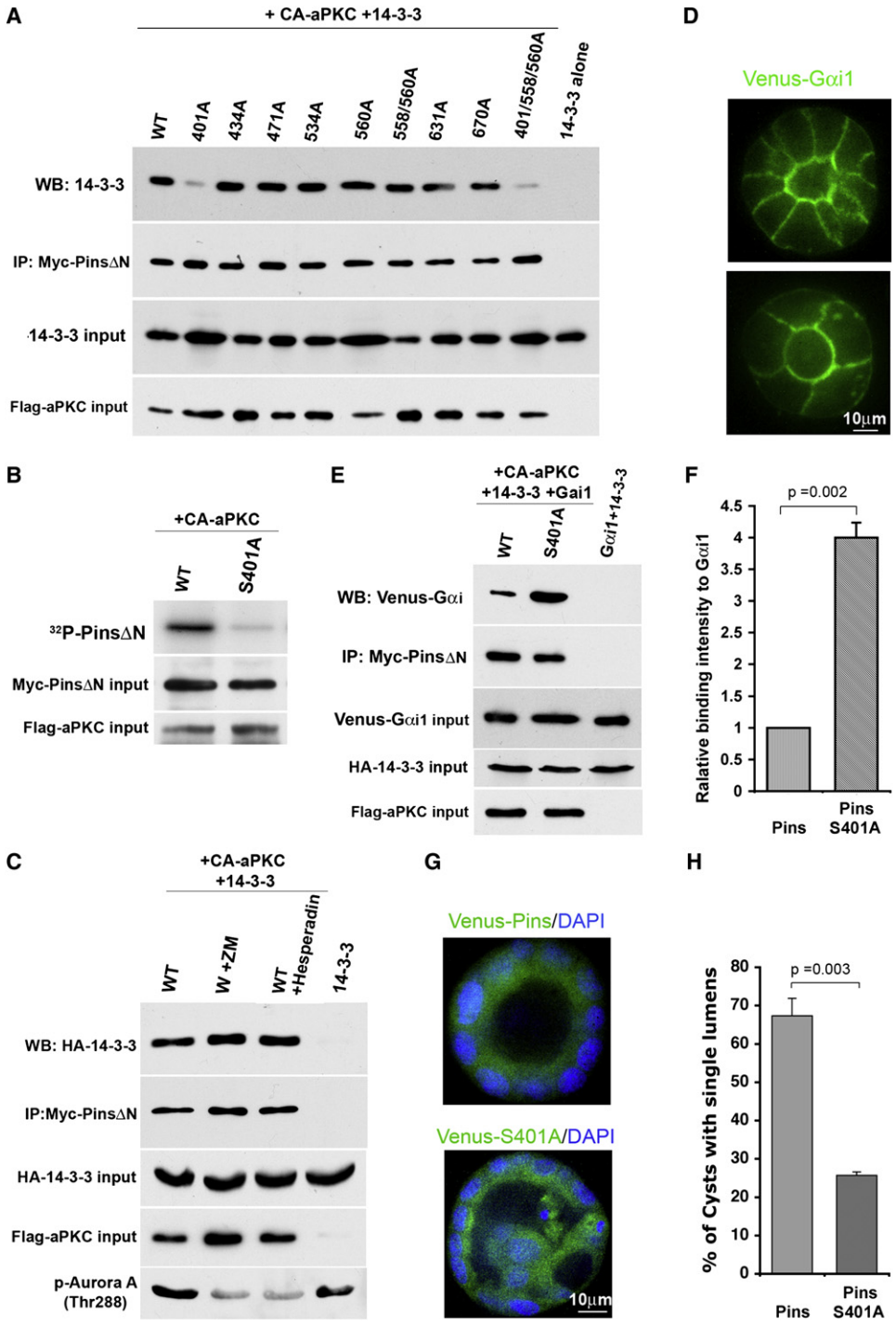

Figure 4. Pins Is Phosphorylated on Ser401 by aPKC, which Regulates Gαi Binding and Lumen Formation

(A) Binding of 14-3-3 to Pins requires phosphorylation of S401 but not of other phosphorylation sites.

(B) Mutation of S401 to A reduces aPKC-dependent incorporation of ^{32}P into Pins.

(C) Aurora kinase inhibitors do not block 14-3-3 binding to Pins.

(D) Venus-Gαi is enriched on the apical and lateral cortex of MDCK cells in cysts.

(E and F) The S401A mutant of Pins binds to Venus-Gαi more robustly than the wild-type Pins.

(E) Myc-Pins ΔN was coexpressed with HA-14-3-3 and activated aPKC. Cell lysates were mixed with lysates from cells expressing Venus-Gαi. Myc immunoprecipitates were blotted for YFP.

(F) Venus-Gαi binding intensities were quantified, and values were normalized to the intensity of wild-type. Error bars are ± one SD.

(G and H) Expression of the Pins S401A mutant causes lumen defects in MDCK cell cysts (G). Cysts were scored for lumen formation and compared using an unpaired t-test (H). Error bars are ± one SD.

This Ser is conserved between mammals and insects and is phosphorylated in mitotic *Drosophila* S2 cells by Aurora A [27]. We tested whether aPKC might indirectly phosphorylate S401 through the activation of Aurora A, but pretreatment with the Aurora inhibitors ZM447439 or hesperidin had no effect on aPKC-dependent 14-3-3 binding to Pins (Figure 4C). These inhibitors were functional, because they reduced the autophosphorylation of T288 in Aurora A (Figure 4C). Most likely, therefore, the phosphorylation is direct.

The Nonphosphorylatable Pins S401A Mutant Causes Lumen Defects and Binds Constitutively to Gαi

Pins is recruited to the cell cortex by association of the C-terminal GoLoco motifs with Gαi subunits in the GDP-bound state [26]. Gαi isoforms are myristoylated and are constitutively localized to the plasma membrane, but it is unknown whether they are polarized in epithelial cells. We therefore examined the expression of a Gαi1-YFP fusion protein in MDCK cysts, and we found that the protein appears enriched on the apical cortex but is also expressed on the basolateral membranes (Figure 4D). Thus, Gαi does not provide information on polarity, and Pins could in principle be recruited by the G protein to the apical cortex (Figure 4C). Exclusion of Pins might occur if its phosphorylation by apical aPKC and recruitment of 14-3-3 reduces the association with Gαi. To test this hypothesis, we expressed wild-type or S401A myc-Pins ΔN with activated aPKC and 14-3-3 and then added cell lysate containing YFP-Gαi. Immunoprecipitates of the myc-Pins were blotted for YFP. As shown in Figures 4E and 4F, the S401A mutant, which cannot interact with 14-3-3, bound Gαi more robustly than the wild-type protein, consistent with the idea that 14-3-3 reduces the affinity of Pins for Gαi. In addition, we asked whether the coexpression of 14-3-3 would reduce the amount of Gαi immunoprecipitated with Pins from transfected cells. Endogenous 14-3-3 interferes with this experiment, but nonetheless a significant decrease in Gαi binding was detected (Figure S3C).

Taken together, these results predict that the S401A point mutant of Pins should mislocalize over the apical surface during mitosis and thereby cause spindle pole orientation defects and multiple lumen formation. To test this prediction, we expressed either wild-type or S401A YFP-Pins from lentiviruses and examined the cysts after 3–4 days. Strikingly, the S401A mutant caused a strong lumen defect (Figures 4G and 4H; note that Pins is diffusely cytoplasmic in interphase cells). Moreover, in mitotic cells, although wild-type YFP-Pins was excluded from the apical cortex, the S401A mutant was not (Figure 5A). In addition, the point mutant caused a significant defect in spindle orientation (Figure 5B), strongly supporting the idea that phosphorylation of S401 by aPKC is crucial for epithelial cell division in the correct plane.

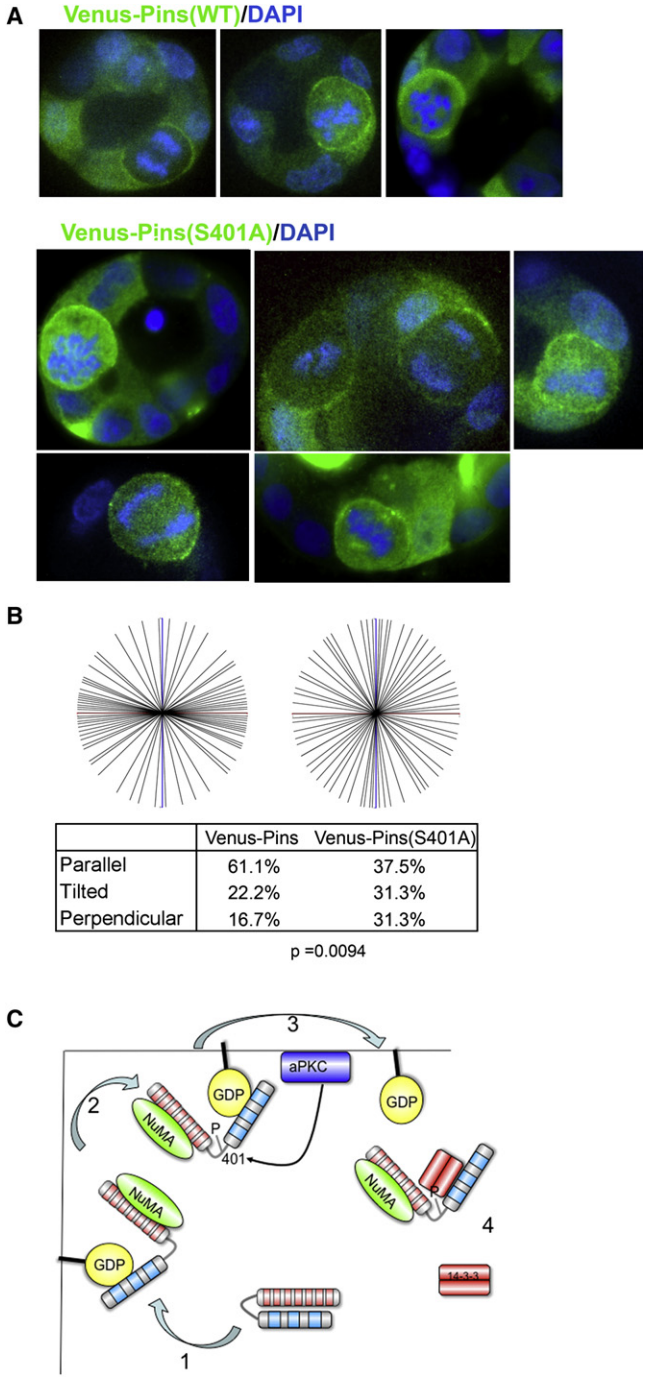

Figure 5. The Pins S401A Mutant Is Mislocalized and Causes Spindle Pole Misorientation in MDCK Cell Cysts

(A and B) Cells were infected with lentivirus expressing wild-type or mutant Venus-Pins and grown as cysts in Matrigel. Mitotic cells were imaged to localize the fusion proteins (A). DAPI staining was used to score the mitotic orientation, and angles were analyzed by Mann-Whitney test (B) (n = 68).

(C) Schematic showing the mechanism by which Pins is excluded from the apical surface. Pins in the closed state is cytoplasmic. During mitosis, it binds NuMA, which switches Pins to an open conformation that can associate with Gαi at the cell cortex. Any Pins bound to apical Gαi can be phosphorylated on Ser401 by aPKC (which is bound to Par6 and activated by Cdc42-GTP). 14-3-3 is recruited to Pins by phospho-Ser401, which displaces Pins from Gαi into the cytoplasm, thereby excluding it from the apical surface.

Discussion

A central question in epithelial biology is how the organization of epithelial sheets is maintained during growth. Cadherin-based intercellular adhesions normally prevent cells from migrating out of the layer, but cells could escape during mitosis unless the spindles are oriented in the same plane as the sheet, so that the daughter cells are retained in the monolayer. Spindle orientation in *Drosophila* stem cells is specified by the Par polarity proteins and by Pins, which by some unknown mechanism attaches astral microtubules to the cell cortex [2, 3, 5]. Recent data have implicated mammalian

Pins/LGN in the orientation of mitosis in epithelial cell division [12], but the mechanism that excludes Pins from the apical surface has not been understood.

We have found that Par3 is an essential upstream component of the epithelial spindle orientation mechanism and acts through recruitment of aPKC to the apical cortex. Importantly, Par3 does not colocalize with apical aPKC in epithelial cells from either mammals or *Drosophila* [21, 27], and the recruitment mechanism has not been fully resolved. However, it is clear that phosphorylation of Par3 by aPKC is necessary for kinase disassociation from Par3; the Crumbs polarity protein and Par6 also play key roles, at least in *Drosophila* [27]. Atypical PKC is activated at the apical cortex by Par6/Cdc42-GTP, where we propose that it can phosphorylate Pins on S401 (Figure 5C). Interestingly, this site is also a target in *Drosophila* for phosphorylation by Aurora A [28], and the phosphorylation is required for recruitment of Dlg and dynein-dependent spindle orientation. However, PhosphoNET (http://www.phosphonet.ca/) predicts that the site might be the target of numerous kinases, including the classical and atypical PKCs.

In interphase cells, Pins is diffusely cytoplasmic and is in its closed conformation, but after nuclear envelope breakdown, NuMA is released and associates with Pins, triggering its switch to the open conformation such that a fraction of the protein can bind to Gαi-GDP at the cell cortex [17]. Any Pins binding to the apical cortex will be phosphorylated, however, by aPKC. We propose that the phosphorylated Pins recruits 14-3-3, which reduces the affinity of Pins for Gαi, so that the apical Pins is released into the cytoplasm (Figure 5C). A similar mechanism has been identified previously for the exclusion of the Par1 polarity protein from the apical surface [24, 25, 29]. Pins on the lateral membrane will not be released and can function to tether astral microtubules, ensuring that mitosis will occur only in the plane of the epithelial sheet. A striking confirmation of this model is the lumen and spindle orientation defects caused in MDCK cell cysts by expression of the Pins S401A point mutant, which acts dominantly over the endogenous protein, as would be predicted if it cannot be excluded from the apical cortex. It will be of interest to determine whether a similar mechanism maintains spindle orientation in the epithelia of *Drosophila* and *C. elegans*, given the high degree of conservation among the polarity proteins involved in this process.

One puzzling feature of our data is that silencing of Par3 results in a shift to a predominantly perpendicular spindle orientation in the cysts, rather than to a random orientation as might be expected if Pins localization is randomized. This might result from the enrichment of Gαi on the apical surface, which in the absence of aPKC activity would recruit more Pins than the lateral membranes. In addition, however, silencing of Par3 expression causes a more pronounced lumen defect than does the silencing of Pins itself [12]. We suspect, therefore, that Par3 and/or aPKC play additional roles in spindle orientation, possibly through effects on Pins-associated proteins.

We have recently discovered that Par3 is essential for normal mammary gland morphogenesis, and that silencing of Par3 in mammary glands results in a loss of aPKC from the apical surface of the luminal epithelium and the accumulation of multiple layers of cells in the ducts [21]. In addition, loss of Par3 increases the proportion of dual-positive progenitor cells in the ducts. We propose that these effects may arise because of defects in the orientation of mitosis by epithelial progenitors, which disrupts the organization of the single luminal epithelial layer and may prevent asymmetric cell divisions of progenitors. Such defects could be important contributors to the invasive behavior of epithelial breast cancers.

Experimental Procedures

Plasmids and Antibodies

Modified versions of lentivector pLVTHM (provided by D. Trono, École Polytechnique Fédérale de Lausanne, Switzerland) were used for stable expression of shRNAs and cDNAs. To generate Venus-NHERF(PB1,2)-aPKC constructs, the PB1 and PB2 domains of *NHERF2* were amplified by polymerase chain reaction to introduce a 5′ BamHI site and 3′ EcoRI site and cloned into pLVTHM-*Venus*. The constitutively active *aPKC* and kinase-dead *aPKC* were then amplified to introduce a 5′ EcoRI site and 3′ SpeI site and cloned downstream of *NHERF2(PB1,2)*. To make Venus-aPKC, wild-type *aPKC*λ was amplified to introduce EcoRI and SpeI and cloned into pLVTHM. *Venus-Pins* and *Venus-Pins S401A* were cloned into pLVTHM through NotI sites. The *Pins* deletion constructs have been described previously [17]. For mass spectroscopy, wild-type *Pins* and *Pins* Δ*N* were cloned into a pK mammalian expression vector containing an S-peptide tag and multiple cloning sites. For mutant screening, Myc-tagged mutants of *Pins* were prepared with a QuikChange XL Site-Directed Mutagenesis Kit (Stratagene).

Anti-β-catenin antibody was from BD Biosciences. Rabbit anti-PKCζ antibody was from Santa Cruz Biotechnology. Mouse anti-α-tubulin was from Sigma-Aldrich. TXR-phalloidin was from Molecular Probes. Rabbit anti-GFP antibody was from Invitrogen. Rabbit anti-phospho-PKCλ and ζ were from Cell Signaling Technology. Mouse anti-ZO1 was generated by D. Goodenough (Harvard Medical School, Boston), and rabbit anti-Par3 was from Millipore (Figure 1) or was a custom antibody generated by Cocalico, Inc. against a peptide (GenScript) coupled to keyhole limpet hemocyanin (Figure 2). Mouse anti-podocalyxin/gp135 was a gift from G. Ojakian (State University of New York Downstate Medical Center, Brooklyn, NY). Myristoylated PKCζ pseudosubstrate inhibitor was from Invitrogen. Transfection methods and hairpin RNA sequences used for silencing Par3 and other genes have been described previously [20].

Cyst Culture in Matrigel and Lentivirus Infection

MDCK cells were trypsinized to produce a single-cell suspension and plated in modified Eagle's medium + 5% fetal bovine serum (FBS) + 2% Matrigel solution in Matrigel-coated eight-well chambers. Lentivirus was produced by transfection of pLVTHM lentivector and packaging system (psPAX2 and pMD2G) into 293LT cells as described previously [21]. To infect cells, we added lentivirus to cells in suspension and grew the cells for 1–2 days before plating cells on Matrigel. Cysts were cultured at 37°C and 5% CO_2.

Immunofluorescence Staining and Microscopy

Cysts were washed twice with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde, and then permeabilized with 0.25% Triton X-100 for 10 min [30]. After blocking with 0.7% gelatin + 0.1% saponin, cysts were incubated with primary antibody in blocking buffer overnight at 4°C. After three washes with blocking buffer, cysts were incubated with Alexa Fluor 488, 546, and 633 for 1–2 hr at room temperature. Cysts were then stained with DAPI for nuclei and mounted in ProLong Gold antifade reagent (Invitrogen). Cysts were imaged with a Yokogawa spinning-disk confocal system (Solamere Technologies) using a 60× NA 1.40 oil-immersion objective lens, a 1K intensified charge-coupled device camera (Stanford Photonics), and InVivo acquisition software (Media Cybernetics). Images were processed with Volocity (PerkinElmer) and assembled with Adobe Photoshop 7.0.

LC-MS/MS Analysis of S-Tagged Pins

S-tagged Pins proteins were expressed in 293LT cells and purified using S-protein agarose beads (Novagen). Mass spectrometric analysis was performed as described previously by Udeshi et al. [31]. Briefly, S-tagged Pins was reduced and carbamidomethylated using dithiothreitol and iodoacetamide, respectively, prior to proteolytic digestion. Peptides amenable to mass analysis were generated using endoproteinase LysC. To achieve optimal sequence coverage, we subdigested a portion of the LysC digest with endoproteinase AspN. The Pins peptides were subsequently loaded on to a C18 column, separated by reverse-phase high-performance liquid chromatography, and analyzed on a front-end ETD-enabled high-resolution LTQ-FT mass spectrometer. The raw data were searched against the human

Pins sequence (NCBI accession number AAN01266) using Open Mass Spectrometry Search Algorithm (OMSSA) version 2.1.1. The CAD (b- and y-type ions) and ETD (c- and z-type ions) data sets were searched using the following parameters: ±0.01 Da precursor mass tolerance, ±0.35 Da fragment ion mass tolerance, protease specificity of LysC, protease specificity of AspN, or no specificity with three possible missed cleavages where applicable. Variable modifications included were carbamidomethylation of Cys, oxidation of Met, and/or phosphorylation of Ser, Thr, and Tyr in addition to GlcNAcylation of Ser and Thr residues. OMSSA performed the removal of reduced-charge species from ETD data sets prior to searching. All site assignments were manually validated.

Measurement of Mitotic Spindle Pole Angles

To measure spindle orientations, lentiviruses were added to cells immediately before plating cells on Matrigel and fixed after 3–4 days. Using this approach, lumens begin to form before silencing has occurred, and most cysts retain single lumens for at least 3 days, which facilitates spindle analysis. Confocal sections of cysts containing mitotic spindles at different phases of mitosis were collected. Spindle angles between the spindle axis and the monolayer surface were measured with ImageJ, for sections of mitotic cells in which both spindles were visible, or both sets of daughter chromosomes. Data were analyzed by nonparametric Mann-Whitney test with Prism software. An unpaired, two-tailed t test gave similar p values.

In-Cell [^{32}P]Phosphate Labeling and Immunoprecipitation

MDCK cells or 293LT cells were transfected with Myc-tagged Pins in the presence of different forms of aPKC or aPKC pseudosubstrate inhibitor, or transfected with Myc-tagged Pins and Pins S401A. After 36 hr growth, cells were washed with phosphate-free medium containing Dulbecco's modified Eagle's medium + 10%FBS. [^{32}P]phosphate (American Radiolabeled Chemicals, Inc.) was then added to a final concentration of 0.2 mCi/ml in phosphate-free medium. After 2 hr, cells were treated with 20 nM calyculin A for 10 min. Cells were then washed with cold PBS and lysed in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate [pH 7.2], 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, 1 μM microcystin-LR, 0.5 mM β-glycerophosphate, protease inhibitor aprotinin, pepstatin, leupeptin, and 1 mM PMSF). Immunoprecipitation was performed using Protein G Sepharose 4 Fast Flow beads (GE Healthcare) conjugated with monoclonal 9E10 antibody (for Myc tag). [^{32}P]phosphate labeling of bound proteins was detected by SDS-polyacrylamide gel electrophoresis and exposure to X-ray film.

Supplemental Information

Supplemental Information includes three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2010.09.032.

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