Aberrant endocytosis leads to the loss of normal mitotic spindle orientation during epithelial glandular morphogenesis

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Epithelial cells form tissues with many functions, including secretion and environmental separation and protection. Glandular epithelial tissues comprise cysts and tubules that are formed from a polarized, single-epithelial cell layer surrounding a central, fluid-filled lumen. The pathways regulating key processes in epithelial tissue morphogenesis such as mitotic spindle formation are incompletely understood, but are important to investigate, as their dysregulation is a signature of epithelial tumors. Here, we describe a signaling axis that manifests in a defect in mitotic spindle orientation during epithelial growth and cystogenesis. We found that activation of the small GTPase ADP-ribosylation factor 6 (ARF6) results in the sustained internalization of cell–surface components such as the cMet receptor and the cell–adhesion molecule E-cadherin. The spindle orientation defect arising from elevated levels of ARF6–GTP required an increase in cMet endocytosis, but was independent of E-cadherin internalization or elevated extracellular signal-regulated kinase (ERK) activity resulting from internalized receptor signaling on endosomes. Misorientation of the mitotic spindle resulted in the development of epithelial cysts with structural abnormalities, the most conspicuous of which was the presence of multiple intercellular lumens. Abnormal mitotic spindle orientation was necessary but insufficient to disrupt glandular development, as blocking the strong prosurvival signal resulting from ERK hyperactivation yielded structurally normal cysts despite continued manifestation of spindle orientation defects. Our findings highlight a previously unknown link between ARF6 activation, cMet receptor internalization, and mitotic spindle orientation during epithelial glandular morphogenesis.

The growth of multicellular organisms relies on the development of highly organized tissues with specialized functions. Within these organisms, epithelial cells form tissues which specialize in a host of functions including secretion, environmental segregation and protection, and sensation. These epithelial cells comprise the basic building blocks of glandular epithelial tissues wherein they form the polarized, single cell layer that surrounds a clear, fluid-filled lumen (1). In vivo epithelial sheets organize to form spherical cysts and cylindrical tubules, the structural units of glandular tissue. Cysts and tubules are topologically equivalent, each cell within these units maintaining apical–basal polarity, with the apical surface facing the lumen, the basal domain adhering to underlying basement membrane, and the cell–cell interactions occurring through the lateral membrane (2). Morphogenesis of this type is heavily dependent on well-ordered proliferation, differentiation, and spatial distribution of cells.

Madin-Darby canine kidney (MDCK) cells can be grown in 3D organotypic culture to form highly polarized cysts (2, 3). Using this system we have previously reported on the development of aberrant glandular structures when epithelial cells are grown with sustained activation of ARF6. Constitutive and sustained ARF6 activation leads to the disassembly of cell–cell contacts followed by internalization of both E-cadherin and growth factor receptors that then accumulate in signaling endosomes. These signaling endosomes can then serve as platforms for hyperactive ERK signaling, which ultimately results in the formation of epithelial cysts with multiple lumens that contain surviving, matrix-deprived cells (4).

Proper development of glandular architecture is achieved through the combination of intercellular adhesion and tight control over mitotic spindle orientation to maintain tissue identity (5). During mitosis, spindle poles align such that the astral microtubules contact the lateral cortex allowing the cells to divide symmetrically within the plane of growth (5). Dividing in this manner places the division plane between the cells and normal to the growth surface. Structurally, this ordered mitosis maintains the identity of both daughter cells and the tissue as a whole, allowing the daughter cells to inherit all three basic membrane domains (apical, lateral, and basal) and to remain adhered to the underlying substrate.

In epithelial cells the loss of mitotic orientation has been coupled to perturbations in E-cadherin function (6). The loss of proper spindle orientation in simple planar epithelia results in cells dividing and growing out of the monolayer. In 3D basement membrane cultures of epithelial cysts, the loss of mitotic orientation can impact glandular morphogenesis. In cultures of intestinal epithelium, for example, the loss of Cdc42 led to unbiased cell divisions and the formation of cysts with multiple intercellular lumens-cysts bearing...
remarkable structural similarities to those found in MDCK cysts upon constitutive ARF6 activation (4, 7). Subsequent investigations have repeatedly demonstrated that the loss of mitotic spindle orientation in 2D cultures leads to the development of multilumen cysts (8–13).

Here we sought to investigate the cellular basis of multiple lumen formation in epithelial cysts downstream of ARF6 activation. We show that unregulated ARF6 activity leads to the loss of mitotic spindle orientation in MDCK cells and in 2D cultures; this results in the formation of abnormal glandular cysts with multiple lumens. Further, we have demonstrated that this shift in spindle orientation is independent of E-cadherin endocytosis or alterations in Cdc42–GTP, both known to promote the loss of normal spindle orientation (7). It is, however, dependent on the endocytosis of a cortical cue, as blocking dynamin-dependent endocytosis will restore the spindle angle and glandular morphology. Furthermore, activation of Rab35-regulated recycling restores normal mitotic spindle orientation together with a corresponding reduction in intracellular receptor levels. As the misorientation of spindle poles is a signature of some epithelial tumors, understanding the underlying cellular basis of this cellular abnormality is of high significance (5).

Results

Constitutive activation of ARF6 in epithelial cells causes a spindle orientation defect

Given the multiple lumen phenotype induced by constitutive ARF6 activation in glandular cysts and the link between this phenotype and mitotic spindle orientation (4, 7), we sought to examine mitotic spindle orientation upon sustained ARF6 activation epithelial cells. To this end, we used the previously described MDCK cell line that stably expresses the ARF6–GTP mutant, ARF6–Q67L, under the control of a tetracycline-responsive transactivator (hereon referred to as MDCKARF6–GTP) (14). Examination of the mitotic spindle angle in these cells revealed a robust defect in biased cell division. Unlike the parental control cells, which had a tightly regulated spindle angle almost universally within 10° of parallel to the growth surface (mean = 4.2°), MDCKARF6–GTP cells showed no significant grouping and an average spindle angle of 39.6° (Fig. 1, A–C and E). This spindle orientation defect was not because of stable incorporation of the constitutively active mutant of ARF6 as we saw similar results when treating parental cells with hepatocyte growth factor (HGF), also known to increase endogenous ARF6–GTP levels (Fig. 1, D and E) (15). Because many of the proteins previously identified as playing a role in the maintenance of mitotic spindle orientation are components of the polarity complex, apical–basal polarity was assessed through the localization of two basolateral markers (β₁-integrin and E-cadherin) and the apical marker gp-135. In the presence of ARF6–GTP, the basolateral markers β₁-integrin (Fig. 1F) and E-cadherin (Fig. 1G) remained localized to the appropriate membrane domains. Gp-135 was exclusively localized to the apical surface of both MDCK and MDCKARF6–GTP cells (Fig. 1, F and G, red). Thus, expression of constitutively active ARF6 did not disturb the polarity of individual cells even if orientation of the mitotic spindle was perturbed.
Spindle orientation defects are caused by increased endocytosis associated with ARF6 activation

A key cellular role for ARF6 is regulation of endocytic membrane trafficking (16), and we have previously documented that inhibiting endocytosis, even in the presence of constitutive ARF6 activation, is sufficient to rescue glandular morphology (4). These results led us to hypothesize that membrane endocytosis was likely facilitating mitotic spindle orientation in MDCK cells. To test this hypothesis, we first utilized Dynasore, the small molecule inhibitor of dynamin (17). Treatment of MDCKARF6–GTP cells with Dynasore resulted in a significant shift of spindle orientations back toward a parallel alignment with the growth surface, with an average spindle angle of 5.6° (Fig. 2, A and C). We note that potential off-target effects of Dynasore have been recently identified following treatment of triple KO fibroblasts depleted of endogenous Dnm1, Dnm2, and Dnm3 (18). The authors demonstrated alterations to peripheral actin ruffling and fluid-phase endocytosis in triple KO cells. To address the potential for off-target effects of the small molecule inhibitor in our system, we made use of MDCK cells stably expressing the dominant-negative mutant of ARF6, ARF6–T27N (MDCKARF6–GDP). We have previously shown that expression of ARF6–T27N blocks cell surface receptor internalization in MDCK cells via inhibition of dynamin-dependent endocytosis (19).

Treatment of parental MDCK cells with HGF, a potent activator of ARF6, also resulted in a significant shift in mitotic spindle alignment. Conversely, HGF-stimulated MDCKARF6–GDP cells maintained normal mitotic spindle orientation. Again, there was tight grouping of spindle angles with an average shift of 5.1° from parallel (Fig. 2, B and C). This attenuation of spindle misalignment indicates that the HGF-mediated realignment of the mitotic spindle requires subsequent activation of ARF6 and together these results indicate that the defect in spindle orientation seen with ARF6 activity depends on ARF6-regulated, and likely dynamin-mediated, endocytosis.

ARF6–GTP regulates mitotic spindle angles via endocytosis and is independent of ERK activation

Sustained ARF6 activation was previously documented to lead to increased ligand internalization including receptors such as cMet. Signaling through these intracellular receptors on endosomes led to robust and long-lived signaling resulting in high levels of phosphorylated (active) ERK (4). We examined whether the hyperactive ERK signaling emanating from “signaling endosomes” was responsible for the resulting spindle orientation defect. To this end we used the small molecule PD98059, an inhibitor of MEK, the kinase directly upstream of ERK, which readily blocks ERK activation in our model system (Fig. 3A). PD98059 treatment of MDCKARF6–GTP cells failed to restore normal spindle orientation (Fig. 3, B and C). PD98059 cells had a mean spindle angle of 50.1°, virtually indistinguishable from untreated MDCKARF6–GTP cells (Fig. 3C). These results demonstrate that the phospho-ERK signal arising from signaling endosomes is not responsible for the shift in spindle orientation upon ARF6 activation.

ARF6–GTP–induced spindle orientation defect is independent of its role in E-cadherin endocytosis

In addition to receptor internalization, elevated ARF6–GTP in epithelial cells leads to the rapid and persistent internalization of E-cadherin from sites of cell–cell contacts. Indeed, in polarized epithelia, activation of ARF6 results in the disassembly of mature cadherin junctions and the internalization of E-cadherin via dynamin-dependent endocytosis where it appears in punctate, intracellular endosomal compartments (19, 20). Interestingly, cadherin junction stability has previously been implicated in mitotic spindle regulation in mammalian cells, Drosophila, and Caenorhabditis elegans (6). Thus, we questioned whether ARF6–GTP–mediated internalization of E-cadherin was responsible for the mitotic spindle defects in MDCKARF6–GTP cells. To uncouple the internalization of E-cadherin from additional cell surface cargo that is endocytosed in response to ARF6 activation, we utilized Wnt3A stimulation. In previous studies, we demonstrated that Wnt3A pro-
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Figure 3. ARF6–GTP–induced spindle angle defect is independent of sustained ERK activation. A, MDCKARF6–GTP cells were grown to near confluence in the presence of doxycycline (to prevent induction of ARF6–GTP expression) or treated with the MEK inhibitor PD98059 as outlined in “Materials and methods.” Treatment of parental MDCK cells with Wnt3A resulted in a defect in mitotic spindle angle; the average spindle angle increased to 21.7° (Fig. 4, A and B). However co-treatment with PD98059 has no effect on spindle orientation as co-treated cells had a mean angle of 19.1° (Fig. 4, C and D). Thus, the endocytosis of cMet and possibly additional cargo is responsible for the defect in spindle orientation seen with ARF6 activation; however, this is independent of ERK hyperactivation and the internalization E-cadherin.

Rab35-mediated cMet recycling rescues mitotic spindle angle in MDCK cells

Rab35, a member of the Ras superfamily of monomeric G proteins has recently been shown to be a binding partner for the ARF6–GAP, ACAP2, and is also known to participate in cargo transport out of recycling endosomes (22–25). Furthermore, several reports have highlighted the antagonistic relationship between Rab35 and ARF6 wherein high levels of ARF6–GTP result in decreased Rab35 activation (26, 27). We first confirmed that ACAP2 and Rab35 co-precipitated with active ARF6 (Fig. 5A). Next, we observed that expression of constitutively active Rab35, Rab35(Q67L), in MDCKARF6–GTP cells resulted in an average spindle angle of 12.6°, rescuing the spindle angle orientation (Fig. 5, B and C). Further, the Rab35–Q67L expressing cells contained reduced amounts of internal cMet (Fig. 5, D and E). We also found that constitutive activation of Rab35 in MDCKARF6–GTP cells did not alter the intracellular accumulation of E-cadherin (Fig. 5F). Taken together, these results indicate that the disruption of intracellular signaling compartments by triggering the fast recycling of receptors and possibly additional cargoes restores proper mitotic spindle orientation.

ARF6–GTP–induced spindle rotation contributes to the formation of epithelial cysts with multiple lumens

Prior reports have linked the loss of mitotic orientation to development of epithelial cysts with a multilumen phenotype similar to that arising from hyperactive ARF6 (4, 7, 8, 10), leading us to examine the mitotic spindle angle in 3D cultures of MDCKARF6–GTP cells. In line with the results above, ARF6–Q67L expression resulted in a profound defect in mitotic spindle angle when measured relative to the approximate center of mass of the cyst (Fig. 6, A and B). The majority of MDCKARF6–GTP cells were seen dividing such that chromosomes aligned at metaphase were nearly perpendicular (average 77.7°) to the radius of the cyst (Fig. 6, B and D). This is in stark contrast to parental cells in which the metaphase plate was parallel to the cyst radius with an average angle of 18.4° (Fig. 6, C and D).

In light of previous research demonstrating that depletion of Cdc42–GTP results in the formation of multilumen cysts stemming from abnormal mitotic spindle orientation (7, 13), we examined the amount of active Cdc42 in MDCKARF6–GTP cells. We saw no reduction in the amount of Cdc42–GTP in MDCKARF6–GTP cysts (Fig. 6E). Additionally, expression of constitutively active Cdc42 (Cdc42–G12V) in MDCKARF6–GTP cells did not rescue spindle orientation defects (Fig. 6F), nor did overexpression of WT HA–Cdc42 lead to a reorientation of the mitotic spindle in parental MDCK cells (Fig. 6G). These results demonstrate that ARF6 activation alters mitotic spindle orientation in 3D cultures of epithelial cells, contributing to the formation of abnormal glandular structures, and these effects are independent of Cdc42 activity.

ARF6–GTP–induced endocytic defects contribute to mitotic spindle rotation and epithelial glandular disruption

The aberrant endocytosis stemming from elevated levels of ARF6–GTP has a profound effect on the development of epithelial glandular architecture (4), in part, as outlined above, because of the abnormal orientation of the mitotic spindle. As such, we sought to investigate the contribution of signaling endosome formation to mitotic spindle orientation and epithelial cystogenesis. Consistent with previous results, inhibiting dynamin-dependent endocytosis downstream of ARF6 activa-
tion results in the development of structurally normal cysts (Fig. 7A). Examination of the mitotic spindle in inhibited cysts revealed the mitotic spindle in these cells was oriented such that the division plane was in line with the radius of the cyst (Fig. 7B). Compared with untreated MDCKARF6–GTP cysts which had an average angle of 19.2°, Dynasore-treated cysts had an average angle of 80.6° (Fig. 7C), comparable to parental MDCK cells in similar culture conditions (Fig. 6D).

Earlier reports have concluded that the abnormal orientation of the mitotic spindle in 3D cultures was sufficient to cause the development of multilumen cysts (7–11). In light of the results above, we investigated the contribution of ARF6-induced ERK hyperactivity on spindle orientation and epithelial glandular morphogenesis. To this end, MDCKARF6–GTP cells in 3D culture were treated with PD98059, beginning 24 h post induction of ARF6–GTP expression. Although ERK inhibition results...
in structurally normal cysts containing a single, clear lumen (Fig. 7D) it did not restore mitotic spindle orientation. MDCK\textsuperscript{ARF6-GTP} cells treated with PD98059 had an average spindle angle of 14.9°, on par with untreated cells showing an average angle of 18.3° (Fig. 7E). Taken together these results demonstrate the importance of tightly controlled endocytosis and intracellular signaling to epithelial glandular morphogenesis, as blocking the long-lived ERK signal emanating from signaling endosomes is sufficient to rescue glandular architecture even in the presence of abnormal mitotic spindle orientation.

Discussion

Critical questions in cell biology surround the mechanisms by which epithelial cells maintain structural organization throughout growth and morphogenesis (28). One decisive point at which cells could exit the normal epithelial monolayer is during mitosis. During epithelial morphogenesis mitotic spindles are oriented in the same plane as the cell sheet to retain daughter cells within the growing monolayer (29). Disruption of the mitotic spindle has substantial impacts on epithelial morphogenesis, resulting in the development of multilumen cysts. Here, we report for the first time that aberrant endocytosis resulting from ARF6 activation leads to spindle angle defects and subsequently the formation of abnormal glandular structures (Fig. 7F). Importantly, we have demonstrated that it is not the disruption of E-cadherin–based cell–cell contacts, a process well-documented to result from ARF6 activation, that leads to cells escaping the epithelial sheet during mitosis. This is in contrast to a previous report, which concluded that E-cadherin served as a cortical cue to orient the mitotic spindle in MDCK cells (6). Critically, however, the authors demonstrate that the depletion of endogenous E-cadherin by siRNA does not lead to a spindle angle defect. Thus, E-cadherin may serve as a cortical cue; however, it is not necessary for epithelial cells to properly orient the mitotic spindle. This is consistent with our results using Rab35 to restore cMet to the basolateral surfaces of transfected cells examined by confocal microscopy.

Recent studies have uncovered an interesting antagonistic relationship between ARF6 and Rab35. Rab35 was identified as a binding partner of the ARF6 GAP ACAP2, which is responsible for recruiting ACAP2 to ARF6-positive endosomes and places Rab35 upstream of ARF6 (25, 30). However, ARF6–GTP has been shown to bind several Rab35 GAPs of the TBC1D family including TBC1D10A (31), TBC1D10B (32), and TBC1D24 (33). Similarly, the constitutive activation of ARF6 was found to cause a cytokinesis defect because of ARF6-medi-
ated inhibition of Rab35 (32). These experiments have led researchers to conclude that ARF6 must act upstream of Rab35. The interaction between ARF6–GTP and the Rab35 GAPs listed above would indicate that high levels of active ARF6 would reduce the levels of active Rab35 and subsequently impede recycling of Rab35-dependent cargo. This is consistent
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with our findings using Rab35–Q67L, in which the addition of the GTPase-deficient protein liberates cMet that accumulated intracellularly with constitutive ARF6 activation. Additionally, a requirement for Rab35 to maintain cadherins at the cell surface and promote cell–cell adhesion has been reported (26). It is quite likely that by using the constitutively active mutant of ARF6 we are in effect removing a portion of Rab35 by sequencing it via the Rab35–ACAP2–ARF6 interaction.

Our results indicate that increased endocytosis and decreased recycling are necessary for this spindle orientation defect to manifest in the presence of constitutive ARF6 activity. It would appear that ARF6–GTP leads to the internalization of a cortical cue; we propose the possibility of the cMet receptor, the absence of which causes cells to divide in an unbiased manner. Surprisingly, sustained intracellular ERK signaling was found to have no effect on the orientation of the mitotic spindle. What is absolutely crucial, however, is the establishment of a downstream cue to enable cell survival absent adhesion to neighboring cells or the basement membrane. Without the stabilization of the prosurvival signaling resulting from ERK phosphorylation, MDCKARF6–GTP cells fail to form abnormal cysts even in the presence of a spindle angle defect. Thus, we have concluded that abnormal spindle orientation is necessary but insufficient for the formation of multilumen epithelial cysts.

ARF6 has not previously been studied in the context of mitotic spindle orientation. ARF6 has well-documented roles in epithelial junction stability, membrane trafficking, and endocytic regulation. More recently, research has implicated ARF6 signaling in altered epithelial morphogenesis and cell invasion (21, 34, 35). Interestingly, normal ARF6 regulation is emerging as an oncogenic pathway featured among multiple tumor types. In breast cancer, the ARF6 GEF, GEP100, plays a crucial role in regulating cell invasion both in vitro and in vivo (36–38). In colorectal cancers, loss of function mutations in SMAP-1 (stromal membrane-associated protein-1), an ARF6-specific GAP, has been associated specifically with cancers showing microsatellite instability (39). Our results further underscore that ARF6 activity must be tightly regulated in epithelial cells to ensure both junctional and structural stability and that loss of this regulation could lead to the onset of spindle orientation abnormalities and the development of preinvasive tumorogenic structures.

Unexpectedly, our analysis revealed that the mitotic spindle defect seen in MDCKARF6–GTP cells appears to be nonrandom. Rather than lacking any sense of spindle orientation upon induction, the spindles appear to predominantly exhibit a rotation 90° to what is seen in parental cells, leaving the orientation normal to the radius of the cyst. It is possible to attribute this to the cells dividing preferentially along what is often their long axis when a cortical cue is abnormally internalized from the lateral surfaces because of high levels of ARF6–GTP. It is tempting to speculate, however, that given the significant enrichment of actin-based cilia protruding from the free apical surface, there may be a pool of cMet that is resistant to internalization because of its association with the actin cytoskeleton. A similar resistance to internalization has been demonstrated for ErbB2 associated with protrusions in breast cancer cells (40). Future experimentation aimed at teasing apart the differences in RTK and cadherin internalization could shed significant light on this possibility.

Materials and methods

Antibodies and plasmids

The following primary antibodies were used: canine-cMet from R&D Systems (Minneapolis, MN); α-tubulin from Sigma; Cdc42 from Invitrogen; mouse monoclonal anti–E-cadherin, a gift from W. Gallin (University of Alberta, Alberta, Canada); myc-tag from BD Transduction Laboratories; Rab35, total ERK, and phospho-ERK from Cell Signaling Technology (Danvers, MA); ACAP2 from Proteintech Group Inc. (Rosemont, IL); and HA-tag from BioLegend (San Diego, CA). Anti–gp-135 mouse monoclonal was a gift from George Ojakian. Anti–β1-integrin (AlIB2) antibody was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. Mouse HRP secondary antibody was purchased from Cell Signaling Technology. Mouse and rabbit heavy chain–specific HRP conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories. Fluorophore conjugated secondary antibodies and rhodamine phalloidin were purchased from Molecular Probes. Nuclear stains used were DRAQ5, purchased from Alexis Biochemicals (San Diego, CA), and To-Pro3 from Invitrogen.

Cdc42–G12V–GFP was kindly provided by Dr. Philippe Chavrier (Institut Curie, Paris, France). Cdc42–WT–HA was a gift from Linda van Aelst (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Rab35–Q67L–myc and Rab35–S22N–myc were generously provided by Dr. Guangpu Li (University of Oklahoma Health Sciences Center, Oklahoma City, OK).

Cell culture

MDCK cells expressing the constitutively active (Q67L) and dominant negative (T27N) mutants of ARF6 were maintained, and mutant protein expression induced as described previously (14). Briefly, these cells express the HA-tagged ARF6 mutants under the control of the tetracycline repressible transactivator, allowing for precise control over mutant protein expression by switching to tetracycline-free media.

For 2D cultures, slight modifications to previously reported protocols (4) were used. 5 × 10⁵ cells in single cell suspensions were overlayered onto a bed of Matrigel recombinant basement membrane (BD Biosciences) in each well of an 8-well chamber slide (BD Falcon) and grown in assay media consisting of growth media + 2% Matrigel. Mutant ARF6 expression was induced 48 h post seeding by replacing media with tetracycline-free assay media. Assay medium was changed every 72 h. For treatment experiments, cells were grown in the presence of PD98059 (25 μg/ml) purchased from Cayman Chemical (Ann Arbor, MI), replaced every 36 h, or Dynasore (100 mM) purchased from Sigma, replaced every 48 h. For Dynasore experiments assay media contained Nu-Serum (BD Biosciences) in place of FBS.

Immunofluorescence and spindle angle measurement

For 2D immunofluorescence, cells were seeded on 18 mm glass coverslips (5 × 10⁵ cells per coverslip) in complete growth
media. These cells were allowed to grow to between 90 and 100% confluence before mutant protein induction, and subsequent immunofluorescence processing using established protocols (4, 21). When using canine cMet (ca-Cmet) antibody, cultures were fixed in paraformaldehyde then stained using protocols published previously (41).

For 2D spindle measurements, if necessary, MDCK cells were induced 24 h prior to treatment initiation. Treatments were added for 16 h prior to fixation and immunofluorescent staining. Images for spindle angle measurements were obtained using a Nikon microscope coupled to a three-channel Bio-Rad MRC 1024 scanning confocal microscope. For 2D cultures, stacks of images were obtained at 100×, at 1-μm intervals. Images were processed using ImageJ software and angle measurements calculated as reported (7). To measure 2D spindle angles we seeded cysts as described above. 48 h post seeding mutant ARF6 expression was induced (where appropriate) and cells grown for 24 h to allow for plasmid expression. Treatments were added starting at 72 h post seeding. Cysts used for spindle measurements were fixed and stained at 6 days post seeding using previously published methods (4). Spindle measurements were then carried out as previously reported (7).

Western blotting and immunoprecipitation

Cells grown to confluence conditions matching those used for immunofluorescence experiments were lysed in co-immunoprecipitation lysis buffer (150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 20 mM Tris-HCl, pH 7.5, 1 mM EGTA) for 15 min on ice. Just prior to use, fresh mammalian protease inhibitor mixture (Sigma) and phosphatase inhibitor (Amresco, Inc., Solon, OH) were added to complete the buffer. The Triton X-100 insoluble fraction was removed by centrifugation at 18,000 × g for 15 min and the lysates then precleared by incubation with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Dallas, TX) for 1 h at 4 °C. Protein was normalized by BCA assay before being incubated with anti-HA antibody overnight at 4 °C. Protein was then precipitated by addition of fresh Protein A/G PLUS-Agarose before being washed three times in freshly made complete lysis buffer. Bead bound protein was eluted into 1× SDS loading dye (46.875 mM Tris-HCl, pH 6.8, 7.5% glycerol, 1.5% SDS, 0.075% bromphenol blue, and 30 mM DTT), separated by SDS-PAGE, and examined by Western blotting as indicated in Figs. 3, 5, and 6.

Electroporation

MDCK or MDCKARF6–GTP cells were trypsinized and resuspended to form a single cell suspension. 1 × 10⁶ cells/well were centrifuged to remove culture media. These cells were then resuspended in an appropriate volume of Neon resuspension buffer at 5 × 10⁶/ml and mixed with desired plasmid DNA. Cells were electroporated using Neon Transfection System (Invitrogen) according to manufacturer recommendations.

Cdc42–GTP assay

6-day MDCK and MDCKARF6–GTP cysts were isolated from Matrigel using BD Cell Recovery Solution (BD Biosciences) as per manufacturer’s recommendations. Isolated cysts were lysed using PAK assay lysis buffer. To determine the levels of Cdc42–GTP, GST–PAK assay was conducted as described previously (42).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 6.0f or Microsoft Excel version 16.12. Student’s t test was used when comparing two groups with data that appeared to be normally distributed with similar variances. Welch’s correction was applied when variances were unequal or the data sets contained a significantly different number of data points. When comparing multiple treatment groups to a single control and the data were normally distributed, one-way analysis of variance (ANOVA) was performed with Dunnett’s correction for multiple comparisons. When multiple groups were being analyzed and each group was compared with all other groups and the data were normally distributed, we performed a one-way ANOVA with Tukey’s multiple comparisons test.

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