Current Biology, Vol. 14, 1451–1461, August 24, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.08.025

Polarity Proteins Control Ciliogenesis via Kinesin Motor Interactions

Shuling Fan,[1,6] Toby W. Hurd,[1,6] Chia-Jen Liu,[2] Samuel W. Straight,[1] Thomas Weimbs,[5] Elizabeth A. Hurd,[3] Steven E. Domino,[3] and Ben Margolis[1,2,4,*]

[1]Howard Hughes Medical Institute
[2]Department of Internal Medicine
[3]Department of Obstetrics and Gynecology
Cellular and Molecular Biology Program
[4]Department of Biological Chemistry
University of Michigan Medical School
Ann Arbor, Michigan 48109
[5]Department of Cell Biology
Lerner Research Institute and Glickman Urological Institute
The Cleveland Clinic
9500 Euclid Avenue
Cleveland, Ohio 44195

Summary

Background: Cilia are specialized organelles that play a fundamental role in several mammalian processes including left-right axis determination, sperm motility, and photoreceptor maintenance. Mutations in cilia-localized proteins have been linked to human diseases including cystic kidney disease and retinitis pigmentosa. Retinitis pigmentosa can be caused by loss-of-function mutations in the polarity protein Crumbs1 (CRB1), but the exact role of CRB1 in retinal function is unclear.
Results: Here we show that CRB3, a CRB1-related protein found in epithelia, is localized to cilia and required for proper cilia formation. We also find that the Crumbs-associated Par3/Par6/aPKC polarity cassette localizes to cilia and regulates ciliogenesis. In addition, there appears to be an important role for the polarity-regulating 14-3-3 proteins in this process. Finally, we can demonstrate association of these polarity proteins with microtubules and the microtubular motor KIF3/Kinesin-II.
Conclusions: Our findings point to a heretofore unappreciated role for polarity proteins in cilia formation and provide a potentially unique insight into the pathogenesis of human kidney and retinal disease.

Introduction

Epithelial morphogenesis has been an area of intense study and has been a platform for examining the signaling events that regulate polarization. Epithelial polarization proceeds via distinct stages including formation of cellular junctions and segregation of apical and basolateral membrane domains. Polarity proteins (Par) originally identified in a screen for mutations that disrupt polarity of the *C. elegans* zygote [1] are intrinsically linked to and regulate epithelial polarization [2–5]. The Par3, Par6, and aPKC polarity proteins form a trimeric complex that localizes to the tight junction. Recently, we demonstrated that the Par3, Par6, aPKC complex is able to functionally and physically interact with a second set of polarity proteins, the Crumbs3 (CRB3), Protein Associated with Lin Seven 1 (PALS1), PALS1 Associated Tight Junction (PATJ) polarity complex [6], which is also localized to the tight junction. This interaction is mediated by the binding of the PALS1 ECR1 sequence to the PDZ domain of Par6 [7].

In both *Drosophila melanogaster* and humans, the apical transmembrane protein Crumbs has been shown to be crucial for polarization [8, 9]. In humans, Crumbs1 (CRB1) localizes to the inner segment of photoreceptors, and loss-of-function mutations result in retinitis pigmentosa and Leber congenital amaurosis [10]. The rod outer segment (ROS) of photoreceptors is derived from a primary cilium and is generated by intraflagella transport (IFT) of proteins along the cilium axoneme between inner and outer segments [11, 12]. In a murine model with a CRB1 mutation, fragmentation and shortening of the photoreceptor cell outer segments is observed, suggesting a possible role for CRB1 in IFT [13]. Renal epithelia express a homolog of CRB1, CRB3, that localizes to the tight junction as well as the apical domain and is essential for polarization. Cilia are also found in the epithelia of kidney where they arise from the apical surface [14]. Defects in cilia formation in kidney epithelia have been strongly implicated in renal cystic disease [12]. Accordingly, we asked if CRB3 plays a role in epithelial ciliogenesis.

Results

CRB3 Localizes to Cilia of Kidney Epithelia

To determine if CRB3 is required for cilia formation in an epithelial context, CRB3 localization in cultured kidney epithelial cells was examined. In addition to the previously observed apical- and tight junction-localized CRB3, immunolocalization revealed that CRB3 is localized to discreet puncta in the primary cilium of MDCK (Manin Darby canine kidney) and IMCD3 (Inner Medullary Collecting Duct) renal epithelial cells as shown by costaining with acetylated tubulin, a known axonemal constituent of primary cilia (Figure 1A). In addition, CRB3 staining was observed in the primary cilia of rat kidney collecting ducts (Figure 1B). To ensure that the observed CRB3 localization was not a staining artifact, the localization of Myc-tagged CRB3 in MDCK cells was examined. Localization of exogenous Myc-CRB3 was similar to that observed for the endogenous CRB3 (Figure 1C). In contrast, another common marker of the apical surface, gp135, is excluded from the cilia (Figure S1A).

CRB3 Expression Is Required for Ciliogenesis

To determine if CRB3 is essential for cilia formation, CRB3 levels were reduced by siRNA in MDCK cells (Figure 2A), and it was found that reduction of CRB3 expression resulted in the inability of MDCK cells to form cilia

*Correspondence: bmargoli@umich.edu
[6]These authors contributed equally to this work.

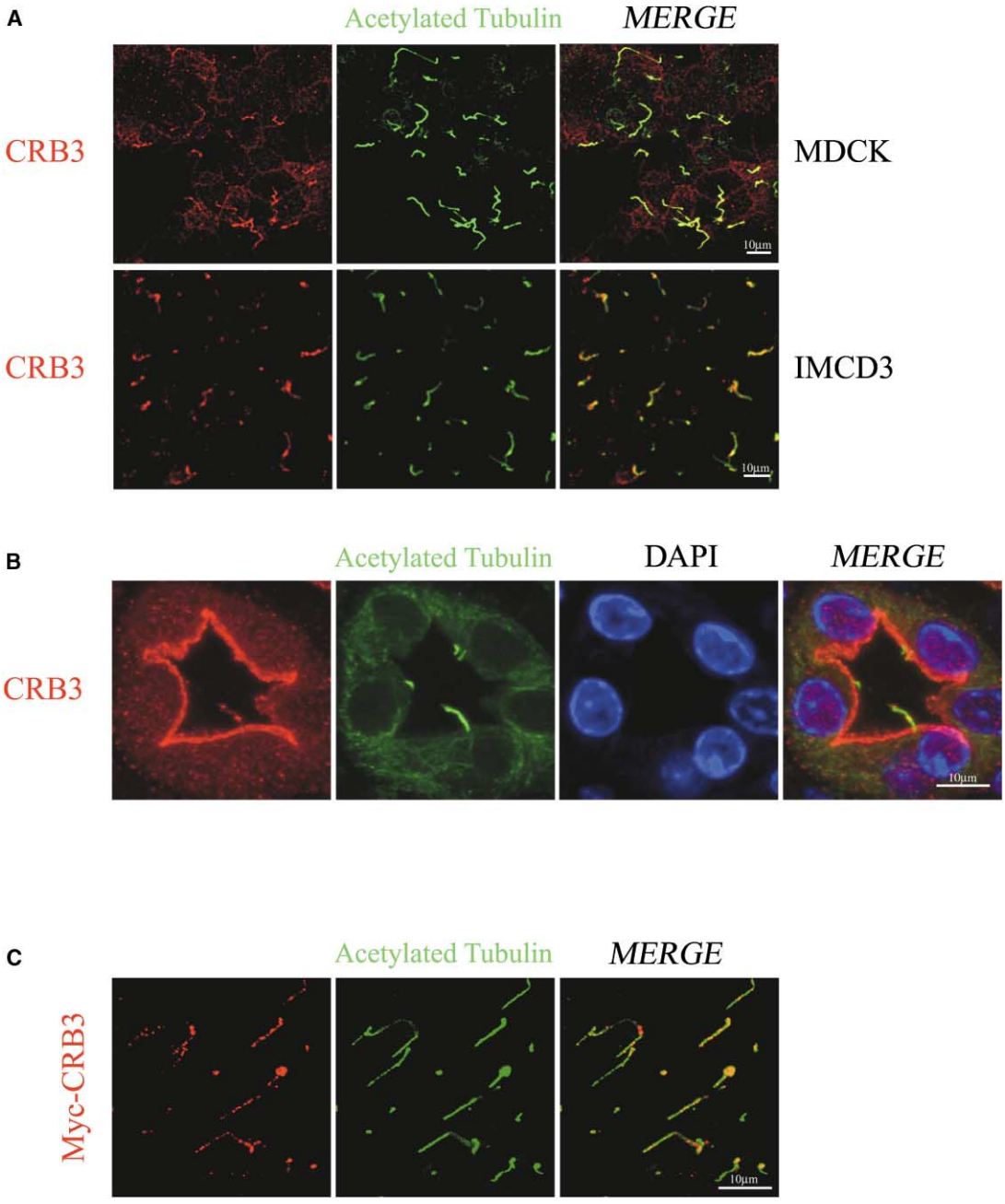

Figure 1. CRB3 Localizes to Primary Cilia

(A) CRB3 localizes to primary cilia of MDCK and IMCD3 cells. MDCK and IMCD3 cells grown for 7 days postconfluence were fixed, permeabilized, and stained with antibodies against the proteins indicated.

(B) Immunolocalization of CRB3 in adult rat kidney section.

(C) Myc-tagged CRB3 localizes to the cilia in MDCK cells. MDCK cells were transfected with Myc-CRB3 and treated as in (A).

(Figures 2B and 2C). Similar results were seen using a second siRNA construct designed against a different region of the CRB3 gene (data not shown). Reduction of the tight junction protein, ZO-1, by siRNA had no effect on cilia formation (Figure S1B). To determine if reduction of CRB3 expression merely resulted in delayed cilia formation, cilia formation was examined in CRB3 siRNA and wild-type MDCK cells over 7 days. In wild-type cells, small cilia were observed as early as 3 days postconfluency; however, cilia formation was completely absent in CRB3 siRNA cells even after 7 days (Figure 2D). These data together suggest that CRB3 expression is required for ciliogenesis.

Par3, Par6, and aPKC Localize to the Cilia

CRB3 has been recently reported to bind directly to the PDZ domain of the polarity protein Par6 [15]. Par6, when complexed with Par3 and aPKC, is involved in the estab-

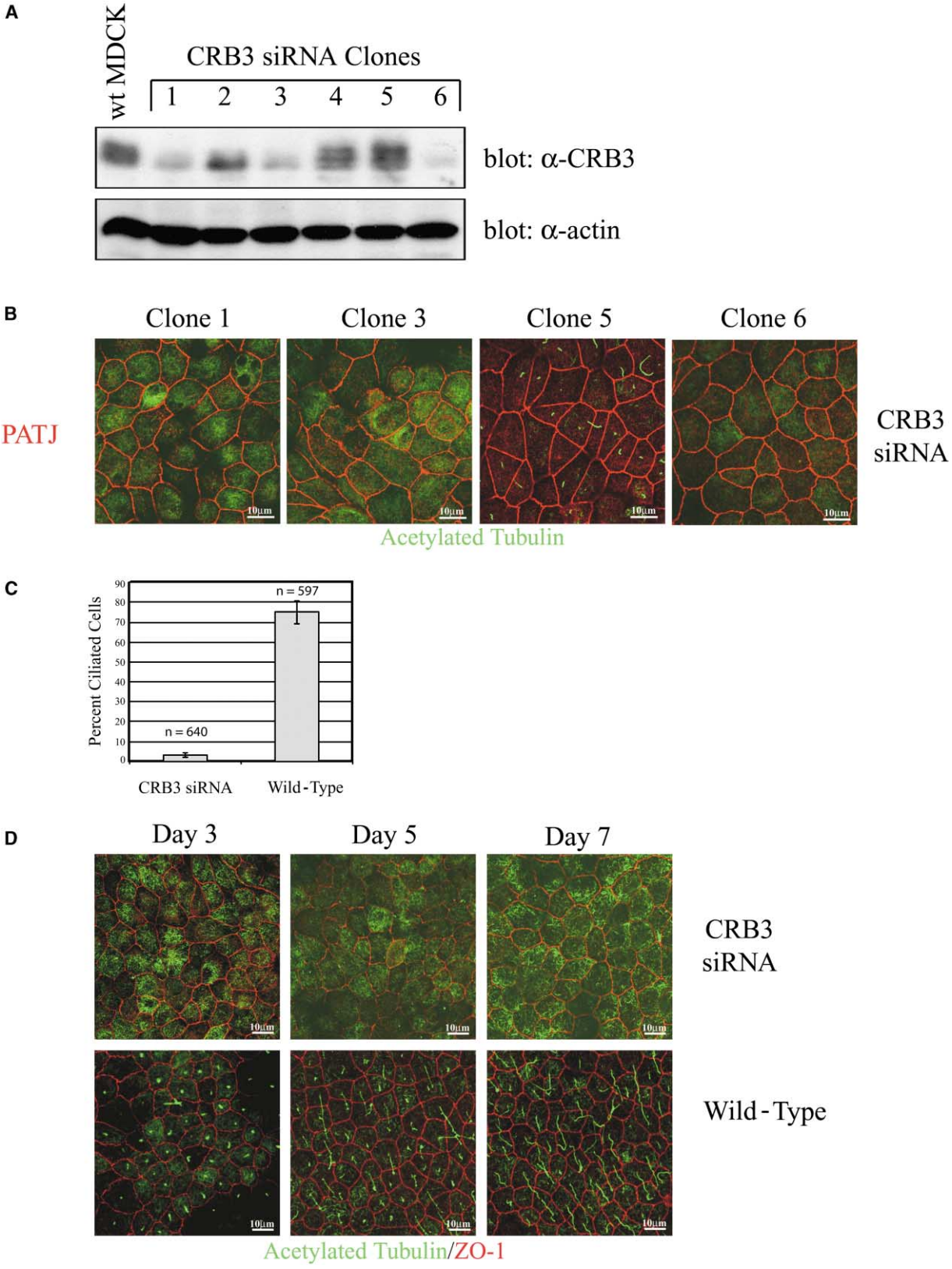

Figure 2. CRB3 Is Required for Cilia Formation

(A) CRB3 expression in MDCK clones stably expressing a CRB3 siRNA construct. CRB3 siRNA clones were lysed and extracts subjected to SDS-PAGE. CRB3 and actin expression levels were detected by Western blotting using the antibodies indicated.

(B) Repression of CRB3 expression prevents cilia formation. CRB3 siRNA clones were grown on filters for 7 days, then fixed, permeabilized, and stained with antibodies against the proteins indicated.

(C) Quantification of ciliated cells in wild-type MDCK cells versus CRB3 siRNA clone 6 cells.

(D) Cilia formation is not just delayed in CRB3 siRNA MDCK cells. Wild-type and CRB3 siRNA clone 6 MDCK cells were grown on filters to confluency and then allowed to grow for the times indicated and treated as in (B).

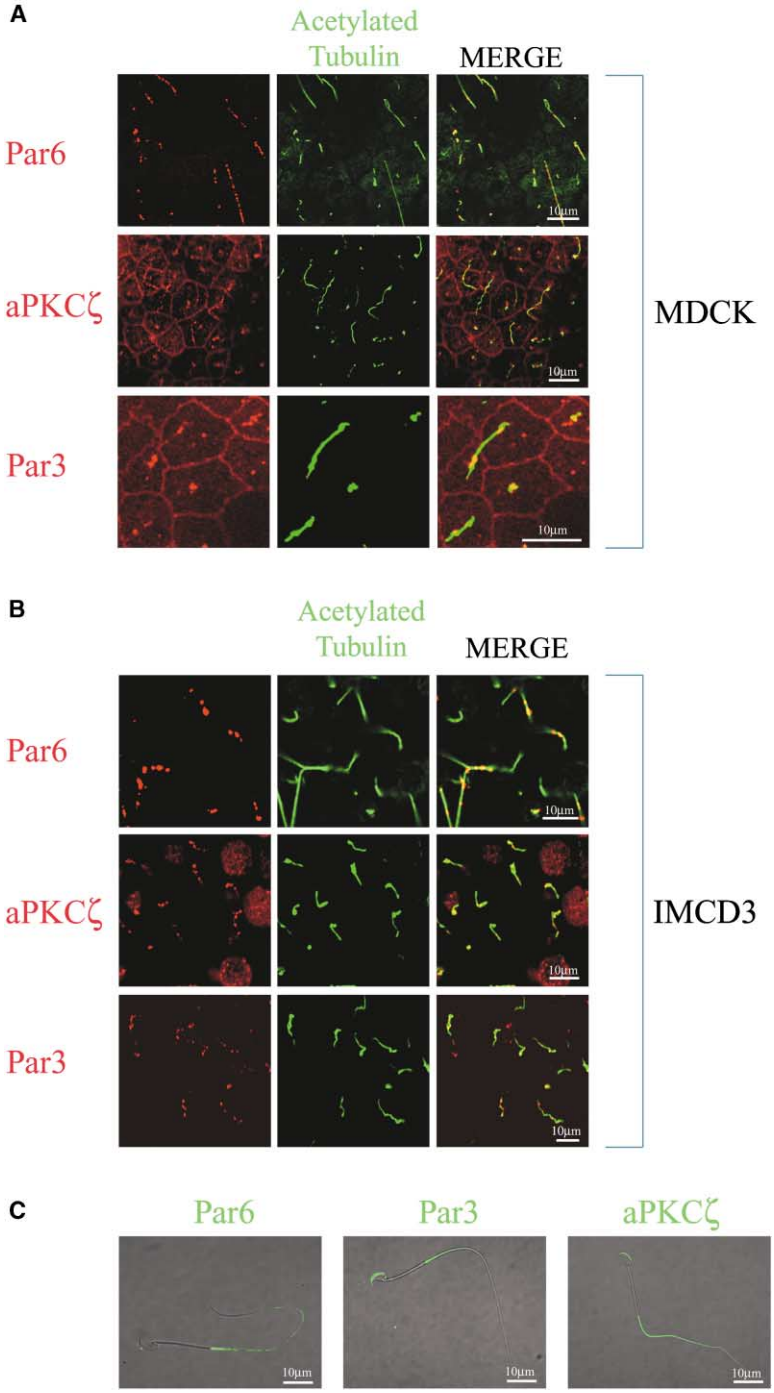

Figure 3. Par3, Par6, and aPKCζ Localize to Both Primary Cilia and Flagella

(A) Par3, Par6, and aPKCζ localize to the primary cilia of MDCK cells. MDCK cells were grown on filters for 7 days postconfluence, then fixed, permeabilized, and stained with antibodies against the proteins indicated.

(B) Par3, Par6, and aPKCζ localize to the primary cilia of murine IMCD3 cells. IMCD3 cells were grown for 8 days and then treated as in (A).

(C) Par3, Par6, and aPKCζ localize to the distal region of sperm flagella. Par3 and aPKCζ also localize to the acrosomal region of the sperm head. Isolated murine sperm were seeded onto slides, fixed, permeabilized, and stained for the proteins indicated. Confocal images are superimposed over differential interference contrast (DIC) images.

lishment and maintenance of epithelial polarity. To address whether CRB3 may regulate ciliogenesis via the Par3, Par6, aPKC polarity complex, we examined whether the Par proteins are able to localize to the primary cilia of cultured epithelia. In MDCK and IMCD3 cells, punctate Par3, Par6, and aPKC localization was observed along the length of the cilium (Figures 3A and 3B, respectively). Recently, two candidate polycystic kidney disease proteins, Polycystin-2 and Polaris, which localize to renal primary cilia, have also been reported to be in the flagella of spermatozoa [16–18]. To determine if the Par3/Par6/aPKC complex is also localized to motile cilia, their localization was examined in isolated murine spermatozoa. Here, Par3, Par6, and aPKCζ all localized to the distal region of the sperm flagellum (Figure 3C). Next, we analyzed whether the polarity proteins were colocalized in the primary cilium. Triple staining revealed that both Par3 and Par6 as well as CRB3 and Par6 colocalize to the same discreet puncta along the length of the cilium (Figures 4A and 4B). Together, these data suggest a conserved role for the Par3/Par6/aPKC complex in both motile and immotile cilia function.

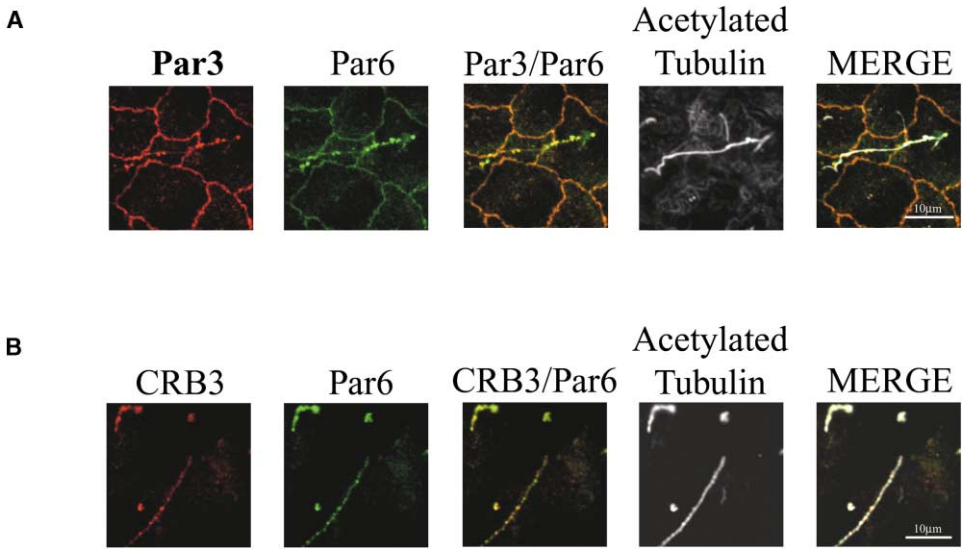

Figure 4. Par6 Colocalizes with CRB3 and Par3 in Cilia

(A) Par3 colocalizes with Par6. MDCK cells were grown on filters for 7 days, then fixed, permeabilized, and stained with antibodies against the proteins indicated.

(B) CRB3 colocalizes with Par6. MDCK cells were treated as in (A).

14-3-3 Proteins Are Important for Ciliogenesis

In *Drosophila melanogaster*, the Par3 homolog Bazooka is localized to the apical domain of follicular epithelia. This localization is maintained by the binding of the phospho-substrate binding protein 14-3-3 (for review see [19]) to Bazooka in conjunction with apical anchoring by Crumbs [20]. In addition, in the *Caenorhabditis elegans* zygote, the 14-3-3 homolog Par5 when depleted gives rise to mislocalization of Par3, Par6, and aPKC [21]. In mammalian epithelia, 14-3-3 binds Par3, and disruption of this interaction results in loss of cell polarity [22]. When examined, we found that the 14-3-3 isoform 14-3-3η localizes to the cilia of MDCK cells in similar fashion to Crb3 (Figure 5A). Overexpression of Myc-14-3-3η did not alter polarity (Figure S1C), and it localizes in a similar fashion to endogenous 14-3-3η (Figure 5B). To assess the role of 14-3-3η in ciliogenesis, MDCK clones stably expressing a 14-3-3η-specific siRNA construct were generated (Figure 5C). In those clones with reduced 14-3-3η expression, ciliogenesis was unable to proceed as in wild-type cells (Figures 5D and 5E). These data suggest that the interaction of Par3 with 14-3-3η is crucial for the function of the Par3/Par6/aPKC complex during ciliogenesis.

Interactions of Microtubules and Polarity Proteins

Next, we examined the mechanism by which the Par3/Par6/aPKC complex localizes to the cilia axoneme. In microtubule sedimentation experiments [23], we found that Par3, Par6, and 14-3-3η could associate with microtubules but only in the presence of AMP-PNP, suggesting that the interaction of these proteins with the cilia axoneme is mediated by a microtubule motor (Figure 6A). As expected, the molecular motor, KIF3a, only associates with microtubules in the presence of AMP-PNP.

The primary anterograde motor in the cilia is the KIF3/Kinesin II complex [12], and kidney-specific knockout of the KIF3a subunit results in loss of cell polarity and the inability of cells to form cilia [24]. The punctate staining observed for Par3, Par6, aPKC, and Crb3 in the primary cilia is similar to that observed for KIF3a (Figure S1D), so we tested whether KIF3a is associated with these proteins. Myc-14-3-3η stably expressed in MDCK cells was used to immunoprecipitate Par3/Par6/aPKC complexes, and it was found that KIF3a efficiently coimmunoprecipitated, implicating the KIF3/Kinesin II complex as the candidate molecular motor that mediates the interaction of Par3,Par6 and aPKC with microtubules (Figure 6B). Furthermore, endogenous Par3 coimmunoprecipitated with endogenous KIF3a (Figure 6C) but not with the Kinesin I complex constituent, Kinesin heavy chain (KHC) (Figure 6D). Examination of the localization of KIF3a and the polarity protein Par6 revealed that they partially colocalize (Figure 6E), indicating that at least some of the cilia-localized KIF3a is associated with the Par3, Par6, aPKC polarity complex.

Atypical PKC Activity Is Important for Ciliogenesis

Signaling through the Par3/Par6/aPKC complex is thought to be regulated by modulation of the kinase activity of aPKC [25, 26]. To assess the role of the Par3/Par6/aPKC polarity complex in the regulation of ciliogenesis, we wanted to selectively depolymerize the primary cilia of epithelial cells and then follow reformation. Previous studies had suggested that cilia may be resistant to the action of microtubule-depolymerizing agents [27]. We found that treatment of ciliated MDCK cells for 3 hr with nocodazole resulted in disassembly of the cilia (Figure 7A) while tight junctions were unaffected, as revealed by retention of contiguous staining of the tight junction marker ZO-1. It was found that 7 hr after removal of nocodazole, cilia reformation could be detected, and after 24 hr, cilia were fully formed. However, addition of

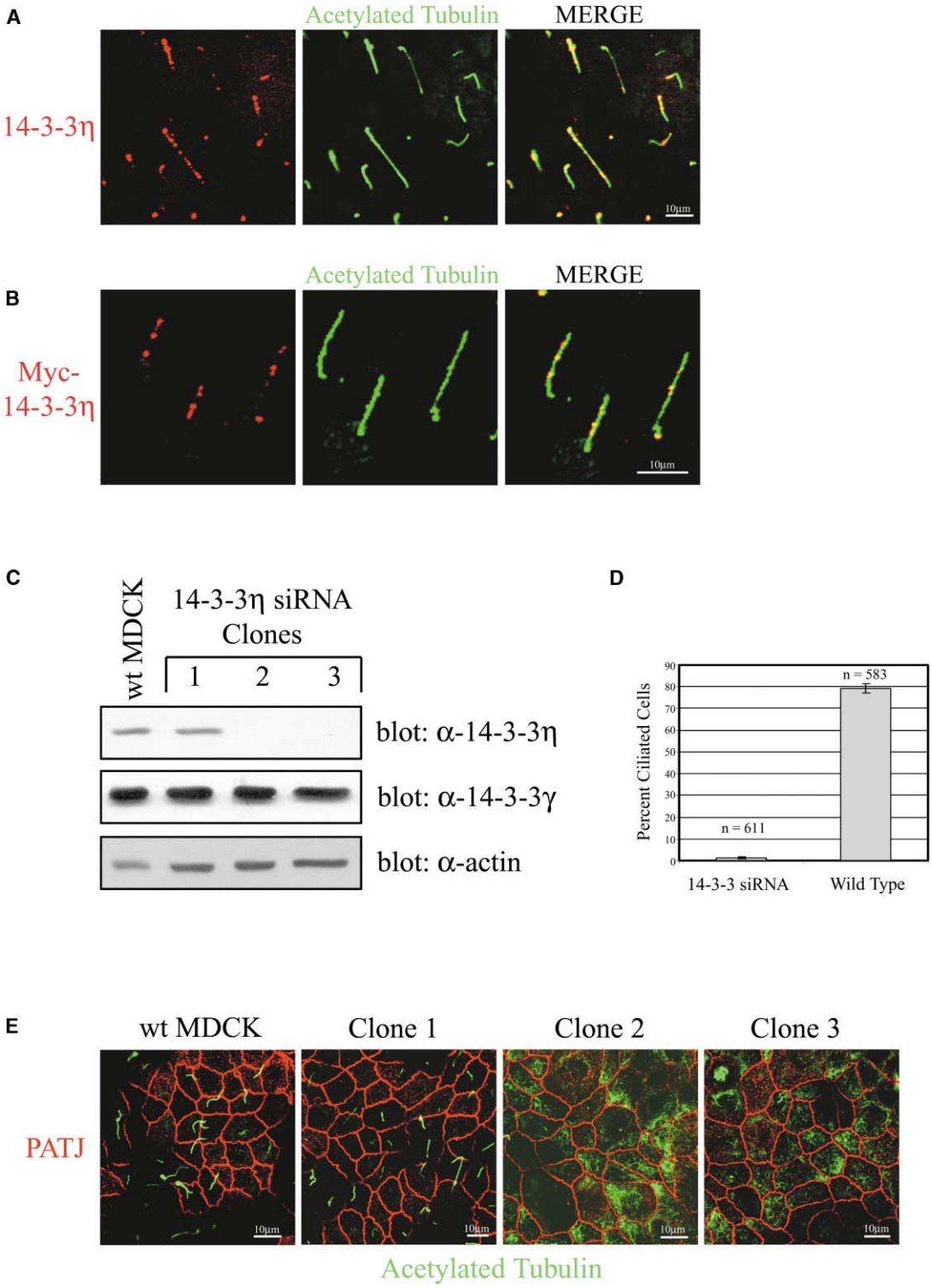

Figure 5. 14-3-3η Localizes to Primary Cilia and Is Required for Ciliogenesis

(A) Endogenous 14-3-3η localizes to the primary cilia. MDCK cells were grown on filters for 7 days, then fixed, permeabilized, and stained with antibodies against the proteins indicated.

(B) Transfected Myc-tagged 14-3-3η localizes to the primary cilium. MDCK cells stably expressing Myc-14-3-3η were treated as in (A).

(C) 14-3-3η expression in MDCK clones stably expressing a 14-3-3η siRNA construct. 14-3-3η siRNA clones were lysed and extracts subjected to SDS-PAGE. 14-3-3η, 14-3-3γ, and actin expression levels were detected by Western blotting using the antibodies indicated.

(D) Quantification of ciliated wild-type and 14-3-3 siRNA clone 3 MDCK cells.

(E) Depletion of 14-3-3η inhibits ciliogenesis. 14-3-3η siRNA clones were grown on filters for 7 days, then fixed, permeabilized, and stained with antibodies against the proteins indicated.

an aPKC-specific inhibitor (Myristolated aPKC pseudosubstrate inhibitor) after nocodazole treatment completely blocked cilia recovery (Figure 7B). These results suggest that aPKC activity is required for microtubule organization during ciliogenesis.

Discussion

This work demonstrates that both the apical determinant CRB3 and the Par3/Par6/aPKC polarity complex are required for ciliogenesis in epithelial cells. In *Drosophila melanogaster*, Crumbs is phenocopied by the Stardust gene whose protein product binds to the carboxyl terminus of Crumbs [28–30]. Similarly in the mammalian tight junction, CRB3 binds and colocalizes with PALS1, the mammalian homolog of Stardust [30, 31]. Tight junction-associated CRB3 interacts with the Par3/Par6/aPKC complex through the PALS1 protein [6, 31, 32], but due to difficulties with antibody staining, it is not clear if PALS1 is localized to the cilia. However, in mammalian epithelia, the CRB3 carboxyl terminus may be able to directly bind to the PDZ domain of Par6 [15]. Furthermore, either direct binding of CRB3 to Par6 or indirect binding via PALS1 provides a mechanism by which the KIF3 complex can traffic CRB3-containing particles into the developing cilium. During ciliogenesis, there is a requirement for the targeted delivery of cilia-specific proteins such as the polycystin 1 and 2 proteins, crucial for the sensory function of the mature cilium, via IFT [12]. It is tempting to speculate that CRB3 connects to IFT particles via the Par3/Par6/aPKC and Kinesin II complexes. The finding of punctate staining for these polarity proteins supports this hypothesis, but more studies will be required to prove this conjecture.

The Par3/Par6/aPKC complex is unlikely to merely provide a protein scaffold that serves to couple Crb3 particles to the KIF3 motor, but it is probable that it also acts to regulate loading onto microtubules and/or processivity. Consistent with this hypothesis, it has been demonstrated in 3T3-L1 adipocytes that activation of aPKCλ is required for loading of KIF3a onto microtubules and the subsequent exocytosis of GLUT4-containing vesicles [33]. This may be similar to the scaffolding of the JNK kinase pathway to classical kinesin [34, 35]. These findings seem to provide a novel insight into how the Par3/Par6/aPKC may control cell polarization in a large number of cell types. In agreement with this, it has recently been shown that Par3 also is able to interact with KIf3a and regulate neurite polarization [36]. By binding to motors and microtubules, this complex can regulate the polarized trafficking of proteins along microtubules. In addition, the Par3, Par6, aPKC complex may function to regulate the stability of the ciliary axoneme. This is supported by the observation that in migrating astrocytes, Par6 and aPKC regulate GSK3b to induce the binding of the APC protein to the plus ends of microtubules [37]. Furthermore, a second plus end-localized protein, EB-1, which localizes to the flagella tip of *Chlamydomonas reinhardtii* [38], regulates microtubule polymerization in conjunction with APC [39].

Our data also demonstrates an important role for CRB3 in epithelial ciliogenesis. Cilia are an area of active investigation in human biology due to their association with numerous diseases including renal cystic disease and retinitis pigmentosa. A paralog of CRB3, CRB1 is expressed primarily in retina and brain [40]. Unlike CRB3, CRB1 has a large extracellular domain, but the intracellular domains of CRB1 and CRB3 that interact with polarity proteins are highly conserved. Mutations in CRB1 are implicated in the retinal diseases Leber congential amaurosis and retinitis pigmentosa [10, 40]. It has been suggested that CRB1 may contribute to these disorders due to defects in cell adhesion or formation of the apical surface. However, our results suggest that Crumbs proteins might also play a role in cilia function. Cilia are important in photoreceptors for the transport of proteins such as rhodopsin from the inner segment to the outer segment [41]. Indeed, other proteins mutated in retinitis pigmentosa have been localized to the cilia, and proteins implicated in ciliogenesis are mutated in genetic syndromes that combine renal cystic disease with retinitis pigmentosa [42, 43].

The results presented here have important implications for cilia formation and may lead to new insights into the pathogenesis of human disease. They also point to an important intersection between polarity proteins, microtubules, and microtubular motors. It has been proposed in lower organisms that members of the Par polarity proteins control microtubule dynamics in mediating some of their effects on cell polarization [44–47]. Modulating motor function may control the directional movement of proteins along microtubules leading to a polarized distribution of proteins. Further work will be necessary to determine if our findings linking polarity proteins to ciliogenesis have implications for the genesis of apical basal polarity.

Experimental Procedures

Antibodies

The following antibodies were used: rabbit anti-PKCζ and anti-Par3 (Upstate, Charlottesville, VA); mouse anti-acetylated tubulin, mouse anti-KIF3a, and rabbit anti-actin (Sigma Aldrich, St. Louis, MO); rabbit anti-14-3-3γ, goat anti-14-3-3η, and anti-Par6 (Santa Cruz Biotech, Santa Cruz, CA); and rabbit anti-14-3-3η (Assay Designs Inc., Ann Arbor, MI). Creation of rabbit antibodies against CRB3 and PATJ are as previously described [31].

Cell Culture

MDCK type II cells were cultured as previously described [6]. siRNA and Myc-14-3-3η stable MDCK cells were cultured in media supplemented with 300 μg/ml Hygromycin B (Roche, Indianapolis, IN). IMCD3 cells were cultured in DMEM/F12 media (GIBCO-BRL, Carlsbad, CA) supplemented with 10% FBS, 2.5 mM L-glutamine, 0.2 g/l sodium bicarbonate, 15 mM HEPES, and 0.5 mM sodium pyruvate. For cilia immunostaining, cells were grown for 7 days postconfluence on 10 mm Transwell filters as previously described [6]. For cilia formation assays, MDCK cells were treated for 3 hr with 33 μM Nocodazole. Cells were washed five times in PBS to remove nocodazole and then incubated in the presence or absence of 100 μM Myristolated aPKCζ pseudosubstrate inhibitor (Calbiochem, San Diego, CA) for the desired time.

siRNA

Double-stranded oligonucleotides corresponding (underlined) to portions of canine CRB3 (top strand: 5′-GATCCGCCATCACTGCCATCATTGTTCAAGAGACAATGATGGCAGTGATGGCTTTTTTGGAAA-3′; bottom strand: 3′-GCGGTAGTGACGGTAGTAACAAGTTCTCTGTTACTACCGTCACTACCGAAAAAACCTTTTCGA-5′) and 14-3-

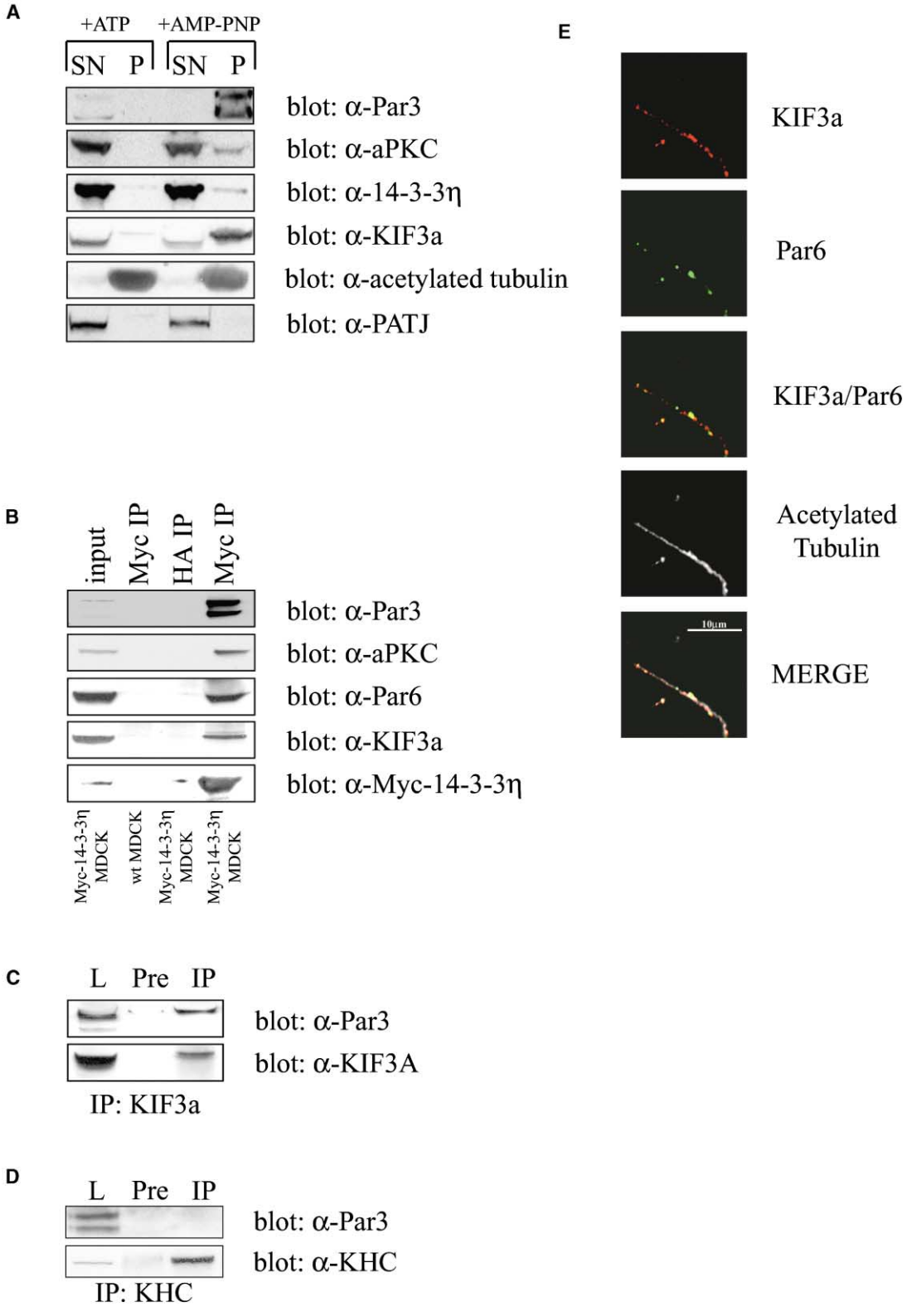

Figure 6. The Par3/Par6/aPKC Polarity Complex Associates with Microtubules in a Microtubule Motor-Dependent Manner

(A) Par3, Par6, aPKC, and 14-3-3η bind microtubules. Prepolymerized microtubules were added to MDCK cytosol in the presence of either ATP or AMP-PNP prior to centrifugation through a sucrose cushion. Supernatants (SN) and microtubule pellets (P) were then subjected to SDS-PAGE and immunoblotted for the proteins indicated.

(B) The Par3/Par6/aPKC polarity complex and the microtubule motor KIF3a coimmunoprecipitates with Myc-14-3-3η. Myc-14-3-3η was immunoprecipitated from Myc-14-3-3η stable expressing MDCK cells, and immunoprecipitates were subjected to SDS-PAGE and immunoblotted for the proteins indicated.

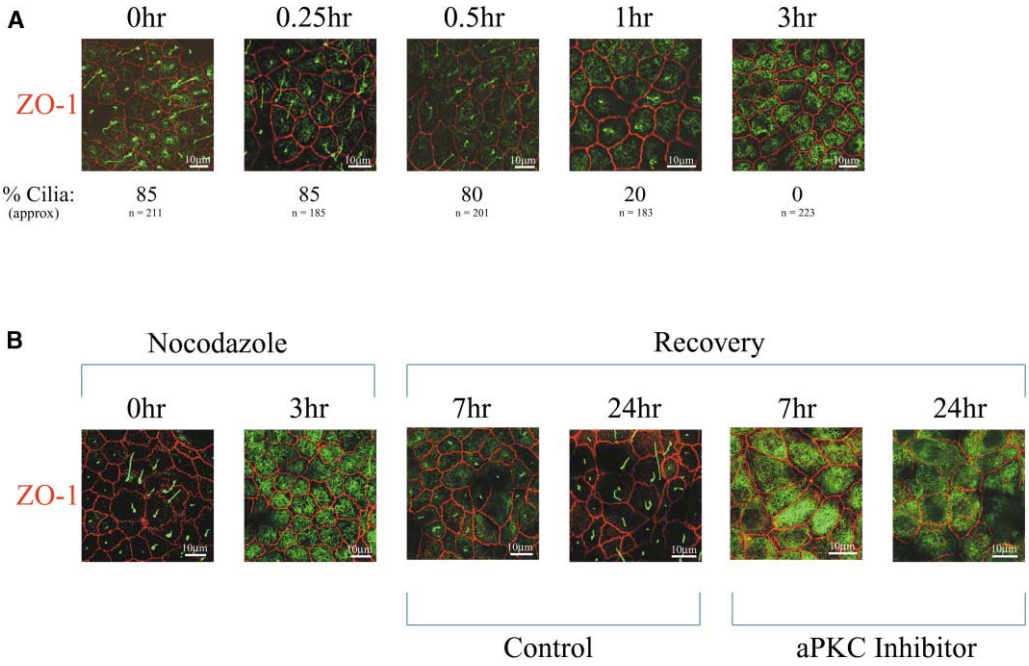

Figure 7. aPKC Activity Is Required for Ciliogenesis

(A) Cilia can be depolymerized by treatment with nocodazole. MDCK cells were grown for 7 days on filters, then treated with 33 μM nocodazole for the times indicated prior to being fixed, permeabilized, and stained with antibodies against the proteins indicated.

(B) MDCK cells were grown for 7 days on filters, then treated with 33 μM nocodazole for 3 hr. Nocodazole was washed out and cells were treated with or without 100 μM aPKC inhibitor for the times indicated.

3η (top strand: 5′-GATCCTGAACCTCTTTCCAATGAATTCAAGAGATTCATTGGAAAGAGGTTCATTTTTTGGAAA-3′; bottom strand: 3′-GACTTGGAGAAAGGTTACTTAAGTTCTCTAAGTAACCTTTCTCCAAGTAAAAAACCTTTTCGA-5′) genes were ligated into the pSilencer vector (Ambion, Austin, TX) and stably transfected into MDCK type II cells under Hygromycin B selection.

Sperm Isolation

Caudal epididymis was dissected from euthanized adult male mice and placed into HTF-HEPES (ZDL Inc., Lexington, KY) + 0.3% Polyvinylpyrrolidone (PVP) for 2 hr at 37°C. Media was aspirated, additional HTF-HEPES was added, and it was incubated for 1 hr at 37°C. Sperm-containing media was separated from cell debris by aspiration and sperm pelleted by centrifugation at 200 × g for 5 min.

Immunoprecipitation and Western Blotting

Immunoprecipitation and Western blotting was performed as previously described [22].

Immunostaining

MDCK and IMCD3 cells grown on Transwell filters were fixed and stained as previously described [6]. Filters were visualized using a Zeiss LSM510 Axiovert 100 M inverted confocal microscope. For sperm immunostaining, sperm were seeded in HTF-HEPES onto polylysine-coated chamber slides (Nalge Nunc, Naperville, IL) at room temperature for 30 min. Sperm were fixed in 4% PFA/PBS for 15 min, permeabilized in 0.1% Triton X100/PBS for 10 min and blocked in 5% BSA/PBS for 1 hr. Sperm were then stained as above. Chamber slides were visualized using an Olympus FV500 inverted confocal microscope. For kidney staining, kidneys from adult Sprague-Dawley rats were used for immunofluorescence microscopy as described previously [48].

Microtubule Spin-Down Assay

Polymerized microtubules were generated as follows. Bovine brain tubulin (Cytoskeleton, Denver, CO) was resuspended in BRB80 (80 mM PIPES [pH 6.8], 1 mM $MgCl_2$, 1 mM EGTA) supplemented with 1 mM DTT and 1 mM MgGTP on ice. 200 nM Taxol (Sigma-Aldrich) was added and the reaction incubated for 5 min at 37°C. 2 μM Taxol was added and the reaction incubated for a further 5 min. 20 μM Taxol was added and the reaction incubated for a further 15 min prior to storage at room temperature. MDCK cells were lysed in buffer A (50 mM HEPES [pH 7.4], 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 0.5% Triton X-100, 1 mM NaF, 1 mM PMSF, and 1 μM each of pepstatin, leupeptin, and aprotinin) and centrifuged at 100,000 × g for 20 min at 4°C. 0.5 mM MgGTP, 5 μM taxol, and either 5 mM MgAMP-PNP or 5 mM MgATP was added to the supernatant, followed by incubation at 33°C for 3 min. In vitro polymerized microtubules and 15 μM taxol were added and incubated at 33°C for 30 min. The extract was layered onto a sucrose cushion (40% sucrose in buffer A, 10 μM Taxol and either 0.5 mM MgATP or 0.5 mM MgAMP-PNP) and spun at 100,000 × g for 30 min at 22°C. Supernatant was retained. The cushion was aspirated with several washes of buffer A and the microtubule pellet was resuspended in SDS-PAGE sample buffer.

(C) Endogenous Par3 coimmunoprecipitates with KIF3a. KIF3a was immunoprecipitated from MDCK extracts. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted for endogenous KIF3a and Par3.

(D) Par3 does not immunoprecipitate with kinesin heavy chain (KHC). KHC was immunoprecipitated from MDCK extracts. Immunoprecipitates were subjected to SDS-PAGE and immunoblotted for endogenous KHC and Par3.

(E) KIF3a and Par6 partially colocalize. MDCK cells were grown on filters for 7 days, then fixed, permeabilized, and stained with antibodies against the proteins indicated. L, lysate; Pre, pre-immune; IP, immunoprecipitate.

Supplemental Data

A supplemental figure can be found at http://www.current-biology.com/cgi/content/full/14/16/1451/DC1.

Acknowledgments

B.M. is an investigator of the Howard Hughes Medical Institute. Confocal microscopy was performed at the Microscopy and Image Analysis Laboratory (MIL), Department of Cell and Developmental Biology, University of Michigan. We would like to thank Kristen Verhey and Gary Smith for useful discussion and Claire Larson for her technical expertise.

Received: April 6, 2004
Revised: July 14, 2004
Accepted: July 16, 2004
Published: August 24, 2004

References

1. Kemphues, K.J., Priess, J.R., Morton, D.G., and Cheng, N.S. (1988). Identification of genes required for cytoplasmic localization in early *C. elegans* embryos. Cell *52*, 311–320.
2. Ohno, S. (2001). Intercellular junctions and cellular polarity: the PAR-aPKC complex, a conserved core cassette playing fundamental roles in cell polarity. Curr. Opin. Cell Biol. *13*, 641–648.
3. Lin, D., Edwards, A.S., Fawcett, J.P., Mbamalu, G., Scott, J.D., and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. Nat. Cell Biol. *2*, 540–547.
4. Petronczki, M., and Knoblich, J.A. (2001). DmPAR-6 directs epithelial polarity and asymmetric cell division of neuroblasts in *Drosophila*. Nat. Cell Biol. *3*, 43–49.
5. Gao, L., Joberty, G., and Macara, I.G. (2002). Assembly of epithelial tight junctions is negatively regulated by Par6. Curr. Biol. *12*, 221–225.
6. Hurd, T.W., Gao, L., Roh, M.H., Macara, I.G., and Margolis, B. (2003). Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. Nat. Cell Biol. *5*, 137–142.
7. Wang, Q., Hurd, T.W., and Margolis, B. (2004). Tight junction protein Par6 interacts with an evolutionarily conserved region in the amino-terminus of PALS1/stardust. J. Biol. Chem. *279*, 30715–30721.
8. Tepass, U., Theres, C., and Knust, E. (1990). crumbs encodes an EGF-like protein expressed on apical membranes of *Drosophila* epithelial cells and required for organization of epithelia. Cell *61*, 787–799.
9. Roh, M.H., Fan, S., Liu, C.J., and Margolis, B. (2003). The Crumbs3-Pals1 complex participates in the establishment of polarity in mammalian epithelial cells. J. Cell Sci. *116*, 2895–2906.
10. den Hollander, A.I., Heckenlively, J.R., van den Born, L.I., de Kok, Y.J., van der Velde-Visser, S.D., Kellner, U., Jurklies, B., van Schooneveld, M.J., Blankenagel, A., Rohrschneider, K., et al. (2001). Leber congenital amaurosis and retinitis pigmentosa with Coats-like exudative vasculopathy are associated with mutations in the crumbs homologue 1 (CRB1) gene. Am. J. Hum. Genet. *69*, 198–203.
11. Pazour, G.J., Baker, S.A., Deane, J.A., Cole, D.G., Dickert, B.L., Rosenbaum, J.L., Witman, G.B., and Besharse, J.C. (2002). The intraflagellar transport protein, IFT88, is essential for vertebrate photoreceptor assembly and maintenance. J. Cell Biol. *157*, 103–113.
12. Rosenbaum, J.L., and Witman, G.B. (2002). Intraflagellar transport. Nat. Rev. Mol. Cell Biol. *3*, 813–825.
13. Mehalow, A.K., Kameya, S., Smith, R.S., Hawes, N.L., Denegre, J.M., Young, J.A., Bechtold, L., Haider, N.B., Tepass, U., Heckenlively, J.R., et al. (2003). CRB1 is essential for external limiting membrane integrity and photoreceptor morphogenesis in the mammalian retina. Hum. Mol. Genet. *12*, 2179–2189.
14. Webber, W.A., and Lee, J. (1975). Fine structure of mammalian renal cilia. Anat. Rec. *182*, 339–343.
15. Lemmers, C., Michel, D., Lane-Guermonprez, L., Delgrossi, M.H., Medina, E., Arsanto, J.P., and Le Bivic, A. (2004). CRB3 binds directly to Par6 and regulates the morphogenesis of the tight junctions in mammalian epithelial cells. Mol. Biol. Cell *15*, 1324–1333.
16. Watnick, T.J., Jin, Y., Matunis, E., Kernan, M.J., and Montell, C. (2003). A flagellar polycystin-2 homolog required for male fertility in *Drosophila*. Curr. Biol. *13*, 2179–2184.
17. Gao, Z., Ruden, D.M., and Lu, X. (2003). PKD2 cation channel is required for directional sperm movement and male fertility. Curr. Biol. *13*, 2175–2178.
18. Taulman, P.D., Haycraft, C.J., Balkovetz, D.F., and Yoder, B.K. (2001). Polaris, a protein involved in left-right axis patterning, localizes to basal bodies and cilia. Mol. Biol. Cell *12*, 589–599.
19. Dougherty, M.K., and Morrison, D.K. (2004). Unlocking the code of 14-3-3. J. Cell Sci. *117*, 1875–1884.
20. Benton, R., and Johnston, D.S. (2003). *Drosophila* PAR-1 and 14-3-3 inhibit Bazooka/PAR-3 to establish complementary cortical domains in polarized cells. Cell *115*, 691–704.
21. Morton, D.G., Shakes, D.C., Nugent, S., Dichoso, D., Wang, W., Golden, A., and Kemphues, K.J. (2002). The *Caenorhabditis elegans* par-5 gene encodes a 14-3-3 protein required for cellular asymmetry in the early embryo. Dev. Biol. *241*, 47–58.
22. Hurd, T.W., Fan, S., Liu, C.J., Kweon, H.K., Hakansson, K., and Margolis, B. (2003). Phosphorylation-dependent binding of 14-3-3 to the polarity protein Par3 regulates cell polarity in mammalian epithelia. Curr. Biol. *13*, 2082–2090.
23. Vernos, I. (2000). Kinesin Protocols, Volume 164 (Totowa, NJ: Humana Press).
24. Lin, F., Hiesberger, T., Cordes, K., Sinclair, A.M., Goldstein, L.S., Somlo, S., and Igarashi, P. (2003). Kidney-specific inactivation of the KIF3A subunit of kinesin-II inhibits renal ciliogenesis and produces polycystic kidney disease. Proc. Natl. Acad. Sci. USA *100*, 5286–5291.
25. Suzuki, A., Ishiyama, C., Hashiba, K., Shimizu, M., Ebnet, K., and Ohno, S. (2002). aPKC kinase activity is required for the asymmetric differentiation of the premature junctional complex during epithelial cell polarization. J. Cell Sci. *115*, 3565–3573.
26. Yamanaka, T., Horikoshi, Y., Suzuki, A., Sugiyama, Y., Kitamura, K., Maniwa, R., Nagai, Y., Yamashita, A., Hirose, T., Ishikawa, H., et al. (2001). PAR-6 regulates aPKC activity in a novel way and mediates cell-cell contact-induced formation of the epithelial junctional complex. Genes Cells *6*, 721–731.
27. Piperno, G., LeDizet, M., and Chang, X.J. (1987). Microtubules containing acetylated alpha-tubulin in mammalian cells in culture. J. Cell Biol. *104*, 289–302.
28. Bachmann, A., Schneider, M., Theilenberg, E., Grawe, F., and Knust, E. (2001). *Drosophila* Stardust is a partner of Crumbs in the control of epithelial cell polarity. Nature *414*, 638–643.
29. Tepass, U., and Knust, E. (1993). Crumbs and stardust act in a genetic pathway that controls the organization of epithelia in *Drosophila melanogaster*. Dev. Biol. *159*, 311–326.
30. Hong, Y., Stronach, B., Perrimon, N., Jan, L.Y., and Jan, Y.N. (2001). *Drosophila* Stardust interacts with Crumbs to control polarity of epithelia but not neuroblasts. Nature *414*, 634–638.
31. Roh, M.H., Makarova, O., Liu, C.J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R., and Margolis, B. (2002). The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. J. Cell Biol. *157*, 161–172.
32. Straight, S.W., Shin, K., Fogg, V.C., Fan, S., Liu, C.J., Roh, M., and Margolis, B. (2004). Loss of PALS1 expression leads to tight junction and polarity defects. Mol. Biol. Cell *15*, 1981–1990.
33. Imamura, T., Huang, J., Usui, I., Satoh, H., Bever, J., and Olefsky, J.M. (2003). Insulin-induced GLUT4 translocation involves protein kinase C-lambda-mediated functional coupling between Rab4 and the motor protein kinesin. Mol. Cell. Biol. *23*, 4892–4900.
34. Verhey, K.J., Meyer, D., Deehan, R., Blenis, J., Schnapp, B.J., Rapoport, T.A., and Margolis, B. (2001). Cargo of kinesin identified as JIP scaffolding proteins and associated signaling molecules. J. Cell Biol. *152*, 959–970.
35. Goldstein, L.S. (2001). Transduction. When worlds collide—trafficking in JNK. Science *291*, 2102–2103.
36. Nishimura, T., Kato, K., Yamaguchi, T., Fukata, Y., Ohno, S.,

and Kaibuchi, K. (2004). Role of the PAR-3-KIF3 complex in the establishment of neuronal polarity. Nat. Cell Biol. *6*, 328–334.

37. Etienne-Manneville, S., and Hall, A. (2003). Cdc42 regulates GSK-3beta and adenomatous polyposis coli to control cell polarity. Nature *421*, 753–756.
38. Pedersen, L.B., Geimer, S., Sloboda, R.D., and Rosenbaum, J.L. (2003). The microtubule plus end-tracking protein EB1 is localized to the flagellar tip and basal bodies in *Chlamydomonas reinhardtii*. Curr. Biol. *13*, 1969–1974.
39. Nakamura, M., Zhou, X.Z., and Lu, K.P. (2001). Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. Curr. Biol. *11*, 1062–1067.
40. den Hollander, A.I., ten Brink, J.B., de Kok, Y.J., van Soest, S., van den Born, L.I., van Driel, M.A., van de Pol, D.J., Payne, A.M., Bhattacharya, S.S., Kellner, U., et al. (1999). Mutations in a human homologue of *Drosophila* crumbs cause retinitis pigmentosa (RP12). Nat. Genet. *23*, 217–221.
41. Wolfrum, U., and Schmitt, A. (2000). Rhodopsin transport in the membrane of the connecting cilium of mammalian photoreceptor cells. Cell Motil. Cytoskeleton *46*, 95–107.
42. Otto, E., Hoefele, J., Ruf, R., Mueller, A.M., Hiller, K.S., Wolf, M.T., Schuermann, M.J., Becker, A., Birkenhager, R., Sudbrak, R., et al. (2002). A gene mutated in nephronophthisis and retinitis pigmentosa encodes a novel protein, nephroretinin, conserved in evolution. Am. J. Hum. Genet. *71*, 1161–1167.
43. Olbrich, H., Fliegauf, M., Hoefele, J., Kispert, A., Otto, E., Volz, A., Wolf, M.T., Sasmaz, G., Trauer, U., Reinhardt, R., et al. (2003). Mutations in a novel gene, NPHP3, cause adolescent nephronophthisis, tapeto-retinal degeneration and hepatic fibrosis. Nat. Genet. *34*, 455–459.
44. Shulman, J.M., Benton, R., and St Johnston, D. (2000). The *Drosophila* homolog of *C. elegans* PAR-1 organizes the oocyte cytoskeleton and directs oskar mRNA localization to the posterior pole. Cell *101*, 377–388.
45. Cox, D.N., Lu, B., Sun, T.Q., Williams, L.T., and Jan, Y.N. (2001). *Drosophila* par-1 is required for oocyte differentiation and microtubule organization. Curr. Biol. *11*, 75–87.
46. Doerflinger, H., Benton, R., Shulman, J.M., and St Johnston, D. (2003). The role of PAR-1 in regulating the polarised microtubule cytoskeleton in the *Drosophila* follicular epithelium. Development *130*, 3965–3975.
47. Labbe, J.C., Maddox, P.S., Salmon, E.D., and Goldstein, B. (2003). PAR proteins regulate microtubule dynamics at the cell cortex in *C. elegans*. Curr. Biol. *13*, 707–714.
48. Li, X., Low, S.H., Miura, M., and Weimbs, T. (2002). SNARE expression and localization in renal epithelial cells suggest mechanism for variability of trafficking phenotypes. Am. J. Physiol. Renal Physiol. *283*, F1111–F1122.