Cdc42 Deficiency Causes Ciliary Abnormalities and Cystic Kidneys

Soo Young Choi,* Maria F. Chacon-Heszele,* Liwei Huang,* Sarah McKenna,* F. Perry Wilson,*† Xiaofeng Zuo,* and Joshua H. Lipschutz*‡

*Department of Medicine and †Center for Clinical Epidemiology and Biostatistics, University of Pennsylvania, Philadelphia, Pennsylvania, and ‡Department of Medicine, Philadelphia Veterans Affairs Medical Center, Philadelphia, Pennsylvania

ABSTRACT

Ciliogenesis and cystogenesis require the exocyst, a conserved eight-protein trafficking complex that traffics ciliary proteins. In culture, the small GTPase Cdc42 co-localizes with the exocyst at primary cilia and interacts with the exocyst component Sec10. The role of Cdc42 in vivo, however, is not well understood. Here, knockdown of cdc42 in zebrafish produced a phenotype similar to sec10 knockdown, including tail curvature, glomerular expansion, and mitogen-activated protein kinase (MAPK) activation, suggesting that cdc42 and sec10 cooperate in ciliogenesis. In addition, cdc42 knockdown led to hydrocephalus and loss of photoreceptor cilia. Furthermore, there was a synergistic genetic interaction between zebrafish cdc42 and sec10, suggesting that cdc42 and sec10 function in the same pathway. Mice lacking Cdc42 specifically in kidney tubular epithelial cells died of renal failure within weeks of birth. Histology revealed cystogenesis in distal tubules and collecting ducts, decreased ciliogenesis in cyst cells, increased tubular cell proliferation, increased apoptosis, increased fibrosis, and led to MAPK activation, all of which are features of polycystic kidney disease, especially nephronophthisis. Taken together, these results suggest that Cdc42 localizes the exocyst to primary cilia, whereupon the exocyst targets and docks vesicles carrying ciliary proteins. Abnormalities in this pathway result in deranged ciliogenesis and polycystic kidney disease.


Cilia are thin rod-like organelles, found on the surface of many eukaryotic cells, with complex functions in signaling, cell differentiation, and growth control. Cilia extend outward from the basal body, a cellular organelle related to the centriole. In kidney cells, a single primary cilium projects from the basal body, is nonmotile, and exhibits an axoneme microtubule pattern of 9+0. In the mammalian kidney, primary cilia have been observed on renal tubule cells in the parietal layer of the Bowman capsule, the proximal tubule, the distal tubule, and in the principal, but not intercalated, cells of the collecting duct.¹

Multiple proteins that, when mutated, result in the development of polycystic kidney disease (PKD) have been localized to renal primary cilia. These include polycystin-1 and -2, the causal proteins in autosomal dominant PKD (ADPKD) (reviewed by Smyth et al.²). Research into pkd2 function in zebrafish has further strengthened the idea that polycystin-2 functions in cilia. Knockdown of pkd2 by morpholino (MO)³⁵ or in mutants⁶ produces phenotypes that are consistent with a role in cilia function, such as curved tails, pronephric cysts, and edema.

Although we are beginning to identify the roles ciliary proteins play in diverse biologic processes, relatively little is known about how these proteins are transported to the cilium.³⁷ The exocyst, originally received December 28, 2012. Accepted April 5, 2013.

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Correspondence: Dr. Joshua H. Lipschutz, Room 405C, Clinical Research Building, 415 Curie Boulevard, Philadelphia, PA 19104.
Email: jhlipsch@mail.med.upenn.edu

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identified in *Saccharomyces cerevisiae*, is a highly conserved 750-kD eight-protein complex known for the targeting and docking of vesicles carrying membrane proteins. It is composed of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (also known as EXOC1–8). Notably, in addition to being found near the tight junction, exocyst proteins were localized to the primary cilium in kidney cells. Sec10 and Sec15 are the most vesicle-proximal of the exocyst components. Sec10 directly binds to Sec13, which, in turn, directly binds Sec4/Rab8, a Rab GTPase found on the surface of transport vesicles. Sec10 then acts as a “linker” by binding the other exocyst components through Sec5. Our previous studies suggested that the exocyst would no longer be able to bind Sec15, and thereby target/dock transport vesicles, without Sec10, and would, instead, disintegrate and be degraded. Importantly, we showed that knockdown of Sec10 in Madin-Darby canine kidney (MDCK) cells abrogated ciliogenesis, while Sec10 overexpression enhanced ciliogenesis. Furthermore, Sec10 knockdown caused abnormal cystogenesis when the cells were grown in a collagen matrix and decreased the levels of other exocyst components and the intraflagellar transport protein 88. This was in contrast to knockdown of exocyst components Sec8 and Exo70, which had no effect on ciliogenesis, cystogenesis, or levels of other exocyst components. On the basis of these data, and its known role in trafficking proteins to the plasma membrane, we proposed that Sec10 and the exocyst are required to build primary cilia by targeting and docking vesicles carrying ciliary proteins.

A possible mechanism to target the exocyst to nascent primary cilia, so it can participate in ciliogenesis, is through the Par complex. We previously showed that the exocyst colocalizes with Par3 and directly interacts with Par6, both components of the Par complex, which also includes atypical PKC. Cdc42 is associated with the Par complex. In addition to their well studied function at cell-cell contacts, the Par complex has been immunolocalized to primary cilia and is necessary for ciliogenesis. The exocyst is regulated by multiple Rho and Rab family GTPases (reviewed by Lipschutz and Mostov), including Cdc42, which regulates polarized exocytosis via interactions with the exocyst in yeast. Using inducible MDCK cell lines that express constitutively active or dominant negative forms of Cdc42, we established that Cdc42 is centrally involved in three-dimensional collagen gel cystogenesis and tubulogenesis. Whether and how Cdc42 might participate in ciliogenesis and cooperate with the exocyst in ciliary membrane trafficking are open questions.

Toward this end, we showed, in cell culture, that Cdc42 co-immunoprecipitated and colocalized with Sec10 and that Cdc42 was necessary for ciliogenesis in renal tubule cells, in that Cdc42-dominant negative expression, small hairpin RNA knockdown of Cdc42, and small hairpin RNA knockdown of Tuba, a guanine nucleotide exchange factor (Gef) for Cdc42, all inhibited ciliogenesis. Exocyst Sec8 and polycystin-2 also no longer localized to the primary cilium, or the ciliary region, after Cdc42 and Tuba knockdown. As noted, we showed that Sec10 directly binds to Par6, and others have shown that Cdc42 also directly binds to Par6. Knockdown of both Sec10 and Cdc42 increased mitogen-activated protein kinase (MAPK) activation.

Here, using two different living organisms, we confirm and extend our *in vitro* findings. We show that *cdc42* knockdown in zebrafish phenocopies many aspects of *sec10* and *pkd2* knockdown—including curved tail, glomerular expansion, and MAPK activation—suggesting, in conjunction with our previous data, that *cdc42* may be required for *sec10* (and possibly *pkd2*) function *in vivo*. Other ciliary phenotypes include hydrocephalus and loss of photoreceptor cilia. We also demonstrate a synergistic genetic interaction between zebrafish *cdc42* and *sec10* for these cilia-related phenotypes, indicating that *cdc42* and *sec10* function in the same pathway. Demonstrating that the phenotypes were not due to off-target effects from the *cdc42* MOs, we rescued the phenotypes with mouse Cdc42 mRNA. Cdc42 kidney-specific knockout mice died of kidney failure within weeks of birth; histologic examination revealed cystogenesis in distal tubules and collecting ducts and decreased ciliogenesis in cyst cells. Cdc42 conditional knockout kidneys showed increased tubular epithelial cell proliferation, increased apoptosis, increased interstitial fibrosis, and MAPK pathway activation, all features of the nephronphthisis form of PKD. These data, along with our previously published results, support a model in which Cdc42 localizes the exocyst to the primary cilium, whereupon the exocyst then targets and docks vesicles carrying proteins necessary for ciliogenesis; if this does not occur, the result is abnormal ciliogenesis and PKD.

**RESULTS**

*cdc42* Is Necessary for Zebrafish Kidney Development

To determine whether *cdc42* might have a role in zebrafish kidney development, we first needed to demonstrate that *cdc42* localized to the kidney. We therefore performed whole mount *in situ* hybridization of zebrafish embryos at 3 days postfertilization (dpf) using antisense (Figure 1A, upper section) and sense (Figure 1A, lower section) probes, which showed that *cdc42* is expressed in the pronephric kidney, eye, and brain of wild-type zebrafish embryos (Figure 1, A and A’).

After localization of *cdc42* to the zebrafish kidney, we then used MOs to knock down zebrafish Cdc42 levels. Per the Zebrafish Model Organism Database (ZFIN), there are three zebrafish isoforms of human Cdc42: isoform A (NP_956926), which is 98% identical; isoform B (NP_001035012), which is 77% identical; and isoform C (NP_956139), which is 91% identical. We chose to knock down *cdc42* isoform A, the most identical, with an antisense start-site MO, which targets both maternal and zygotic transcripts. Injection of 3 ng of the start-site MO against zebrafish *cdc42* isoform A (hereafter called “*cdc42* AUG MO”) into one-cell-staged embryos, compared with the injection control of phenol red only (Figure 1B, left section), revealed multiple defects at 3 dpf and 5 dpf that
Figure 1. *cdc42* expression occurs in the zebrafish kidney, eye, and brain, and *cdc42* knockdown by antisense MOs results in abnormal phenotypes. (A) Lateral views of whole mount in situ hybridization of zebrafish embryos at 3 dpf with antisense (upper part) and sense probes (lower part). *cdc42* is expressed in kidney, eye, and brain. (A') Higher magnification image of the pronephric kidney and tubule highlights *cdc42* expression in this region (arrows). (B) Phenotype of injection control (with phenol red) embryos and *cdc42* morphants (MO) at 3 dpf and 5 dpf. Defects in *cdc42* morphants include smaller eyes (arrow), hydrocephalus (arrowhead), pericardial edema (*), and a short curved tail. (C) *cdc42* protein at 3 dpf was undetectable by Western blot in 3 ng *cdc42*AUG MO embryos. Bar, 200 μm. (D) The *cdc42*AUG MO embryos were rescued by co-injecting mouse mRNA, which is resistant to the *cdc42*AUG MOs, due to a difference in primary base pair structure. The highly significant rescue with mouse Cdc42 mRNA shows that the ciliary phenotypes seen in the *cdc42*AUG MO embryos are not due to off-target effects.
were consistent with ciliary defects, including hydrocephalus, small eyes, pericardial edema, and a short curved tail (Figure 1B, right section). These phenotypic defects are consistent with the cdc42 expression pattern we found by in situ hybridization (Figure 1A).

A typical injection trial of 3 ng cdc42AUG MO resulted in the following phenotypes: 11% wild-type; 28% with edema only; 13% with edema and small eyes; and 48% with edema, small eyes, and hydrocephalus (n = 63). In comparison, injection controls showed no abnormal phenotypes (n = 73). In all the trials combined, the number of abnormal embryos after injection of 3 ng of the cdc42AUG MO, compared with controls, was highly statistically significant at P < 0.001. Supplemental Table 1 lists all the zebrafish experiments and statistical analyses (n = 1877). Cdc42 protein at 3 dpf was undetectable by Western blot in the cdc42 morphant embryos (Figure 1C), indicating that this cdc42 MO effectively knocked down cdc42. Importantly, we rescued the phenotypes with mouse Cdc42 mRNA, which is resistant to the cdc42AUG MO be-


cdc42 Morphants Have Ciliary Defects

We next investigated the morphant phenotypes seen in Figure 1 in more detail by histologic and immunofluorescence studies. Hematoxylin and eosin staining of fixed sections showed defects in the eye (specifically, loss of the outer segments of the photoreceptor cells) and hydrocephalus, an abnormal accumulation of fluid in the ventricles of the brain (arrowhead in Figure 2A). In the eye, the outer segment of the photoreceptor cell, which is a modified cilium,30 was clearly absent when probed using antibody against acetylated α-tubulin, which stains primary cilia. The cilia in the cells surrounding the dilated ventricles appeared intact, although their function was not tested (not shown). We next examined the pronephric kidneys. The morphologic features of the glomeruli were abnormal. Instead of the normal compact U-shaped glomerulus, glomeruli from cdc42AUG MO embryos showed disorganization and expansion (Figure 2C, arrow). The renal tubules were also clearly dilated (asterisk in Figure 2C).

Given the ciliogenesis defects observed with cdc42 knockdown in vitro and in vivo, we predicted that pronephric cilia would be shorter or absent in the kidneys of the cdc42AUG MO embryos. Somewhat surprisingly, pronephric cilia at 27 hours postfertilization appeared of normal length in cdc42AUG MO embryos by immunofluorescence (Figure 2, D–G); however, the cilia were disordered within the medial (and to a lesser degree the posterior) pronephros, where dilations and pronephric cysts have been observed in other zebrafish cilia mutants.6,31 The discrepancy in pronephric ciliogenesis phenotypes between Cdc42 knockdown in vitro and in vivo may be explained by the presence of cdc42 isoforms B and C, although these isoforms were not detected by our antibody (Figure 1C). The disorganized cilia were similar to what we observed in sec10 and pkd2 morphants.29

Also similar to sec10 and pkd2 morphant embryos,3 cdc42AUG MO embryos showed glomerular expansion in the pronephros by in situ hybridization with the glomerular marker, Wilms tumor 1a (wt1a) at 3 dpf. Wild-type embryos showed a condensed glomerular stain (Figure 2, H and H', 100% condensed; n = 30), whereas cdc42AUG MO embryos showed an enlarged stain (Figure 2, I and I'); 18 of 19 embryos with enlarged stain). This difference was highly significant at P < 0.0001.

sec10 and cdc42 Genetically Interact for Cilia-Related Phenotypes

Our in vitro2,18 and in vivo29 analyses together support a link between exocyst sec10 and cdc42. The curved tail, hydrocephalus, small eyes, edema, and wt1a expansion phenotypes shared between sec10MO and cdc42MO embryos have also been observed upon knockdown of other ciliary proteins.32,33 We therefore wanted to directly test for a specific genetic interaction between these two genes. We titrated both sec10 and cdc42 MOs to find suboptimal doses that did not result in strong gross phenotypes on their own. Interestingly, when we co-injected both MOs at these reduced doses, we observed a striking synergistic effect. Co-injection of 2 ng cdc42MO plus 7.5 ng sec10MO yielded curved tail up, hydrocephalus, small eyes, edema, and wt1a expansion phenotypes, while each MO alone produced mostly wild-type phenotypes. Seven separate trials were performed and a total of 1454 embryos were examined. The addition of 7.5 ng sec10MO to 2 ng of cdc42MO resulted in a greater proportion of abnormal phenotypes than expected based on the results of single-agent experiments (P < 0.001), thereby demonstrating genetic synergy (Figure 3, A–D). The addition of 7.5 ng sec10MO to 1 ng of cdc42MO also resulted in a synergistic effect, although the effects were less dramatic (P < 0.001 compared with uninjected controls, and P = 0.058 when compared with 1 ng of cdc42MO alone). The genetic interaction we observed between sec10 and cdc42 MOs suggests that cdc42 plays a role in sec10 function in multiple cilia-related processes. Similar to our previous in vitro findings in Cdc42 knockout cells,18 by Western blot we also found increased levels of phosphorylated (active) ERK (pERK) in most cdc42AUG MO embryos (Figure 3E).

Generation of Cdc42 Kidney-Specific Knockout Mice

Zebrafish have pronephric kidneys and mammals have more complex metanephric kidneys. To determine the role of Cdc42 in metanephrogenesis, we decided to knock out Cdc42 in mice. Global knockout of Cdc42 resulted in early embryonic lethality, so we needed to generate a kidney-specific Cdc42 knockout mouse. Dr. Brakebusch generously allowed us to use his Cdc42fl/fl mice. Cdc42fl/fl mice34 and the targeting scheme are shown in Figure 4A.
Figure 2. cdc42AUG MO embryos show abnormal ciliary development. (A) Hematoxylin and eosin staining of fixed sections shows defects in the eye and hydrocephalus (arrowhead). (B) In the eye, the outer segment of the photoreceptor cell, which is a modified cilium, is absent (compare arrows in control, left, versus cdc42AUG MO embryos, right). Cilia are shown in green after staining with antibodies against acetylated α-tubulin. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer where photoreceptor cells are found. (C) Hematoxylin and eosin staining of fixed sections shows a normal compact U-shaped glomerulus in the controls on the left, with cdc42AUG MO embryos displaying disorganization and expansion of the glomerulus (arrows). The renal tubules are also dilated in the cdc42AUG MO embryos (*). (D–G) Medial and posterior pronephric duct cilia visualized with antiacetylated α-tubulin antibody (green) at 27 hours postfertilization. Compared with cilia in injection controls, mutant cilia are disordered. (H–I) In situ hybridization for the early kidney marker wt1a (with enlarged insets), 3 dpf, dorsal view ×16 magnification. An uninjected embryo with condensed glomerular stain (H and H′) is shown, along with a 3 ng cdc42AUG MO embryo with expansion of the wt1-stained area (I and I′). The increase in glomerular size in the cdc42AUG MO versus control embryos was significant at $P<0.0001$. Bar, 10 μm for all images.
kidney-specific loss of Cdc42 might be deleterious, even at this early age. The examination of 67 pups tested from P11 to P33 confirmed the lethality for the homozygous kidney-specific Cdc42 knockout mice, but not for the heterozygous kidney-specific Cdc42 knockout mice. There were 22 Cdc42\(^{fl/fl}\), 19 Cdc42\(^{fl/+}\), and 24 KspCre;Cdc42\(^{fl/+}\) mice, with 1 dead (mostly eaten) homozygous Cdc42 knockout (KspCre;Cdc42\(^{fl/fl}\)) mouse found at P15, and 1 long-surviving homozygous Cdc42 knockout (KspCre;Cdc42\(^{fl/fl}\)) mouse that was euthanized at P33 because of failure to thrive, growth restriction, and renal failure (BUN, 201 mg/dl) (Figure 5A). The paucity of kidney-specific Cdc42 knockout mice (KspCre;Cdc42\(^{fl/fl}\)) mice after P10, compared with the expected 25% Mendelian ratio, indicates that lack of Cdc42 in the kidneys leads to an early postnatal death. The cause of death in the homozygous kidney-specific Cdc42 knockout mice is almost certainly renal failure, given significant elevations in BUN (Figure 5B). By Western blot of whole kidney lysates, we did not see a significant decrease in Cdc42 in the KspCre;Cdc42\(^{fl/fl}\) mice (Figure 5C), which we attribute to the limited expression of KspCad-Cre in a subset of renal tubule and collecting-duct cells (i.e., most of the cells in the kidneys of KspCre;Cdc42\(^{fl/fl}\) mice express normal amounts of Cdc42). Supporting this interpretation, by immunofluorescence we saw an absence of Cdc42 in cysts in KspCre;Cdc42\(^{fl/fl}\) mice, while normal-appearing tubules had Cdc42 present (Figure 5D). Interestingly, the enhanced expression of Cdc42 on the apical surface of the normal-appearing tubules corresponds to the localization that we\(^{18}\) and others\(^{39}\) have shown in cell culture.

**Figure 3.** cdc42 and sec10 genetically interact. A synergistic interaction resulting in hydrocephalus (arrowhead), small eyes (arrow), pericardial edema (*), and tail defects was observed upon co-injection of suboptimal doses of 2 ng cdc42MO plus 7.5 ng sec10MO (B and B’). Injection of these amounts of these MOs alone did not generally result in an abnormal phenotype (A). Bar, 1 mm. (C) Histograms show the quantification of the effect of MOs at 3 dpf. Co-injection of suboptimal doses of 2 ng cdc42MO and 7.5 ng sec10MO resulted in an increased abnormal phenotype. Seven separate trials were performed and a total of 1,454 embryos examined. The synergistic effect of adding 7.5 ng sec10MO to the 2 ng of cdc42MO was significant at P<0.001. (D and D’) Expansion of the wt1-stained area is seen in the 2 ng cdc42MO plus 7.5 ng sec10MO embryo and is similar to the expansion seen in the 3 ng cdc42MO (compare Figure 3, D and D’, with Figure 2, I and I’). (E) Increased pERK levels were detected in 3 ng cdc42MO embryos. Lysates were generated from 10 embryos in each condition. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; tERK, total ERK.

To knock out Cdc42 specifically in the kidney, we used a kidney tubule cell-specific Cre mouse line, KspCadherin-Cre (KspCad-Cre), which has been previously used to generate kidney tubule cell-specific mutants of ciliary proteins.\(^{35}\) The breeding strategy is shown in Figure 4B. All four possible genotypes were readily and reproducibly determined using a PCR-based genotyping strategy (Figure 4C).

**Kidney-Specific Knockout of Cdc42 Leads to Early Postnatal Death**

KspCre;Cdc42\(^{fl/+}\) mice were backcrossed to Cdc42\(^{fl/fl}\) mice as described in Figure 4B. Of 65 pups tested at E16.5 to P10, there were 15 Cdc42\(^{fl/+}\), 25 Cdc42\(^{fl/+}\), 12 KspCre;Cdc42\(^{fl/+}\), and 13 KspCre;Cdc42\(^{fl/fl}\) mice. The moderate decrease in heterozygous and homozygous Cdc42 kidney-specific knockout mice from the expected 25% Mendelian ratios, suggests that

**Kidney-Specific Knockout of Cdc42 Results in Cysts in the Distal Tubules and Collecting Ducts**

Hematoxylin and eosin–stained sections from the kidneys of Cdc42 kidney-specific knockout mice at P4 and P6 show many small cysts (Figure 6, A–D) that localize to the distal tubule and collecting ducts, as determined by negative staining of cyst cells with lotus tetragonolobus agglutinin lectin, a lectin specific for proximal tubule cells, and positive staining of cyst cells with peanut agglutinin lectin (PNA), a lectin specific for distal tubule and collecting-duct cells (Figure 6, E and F). The localization of the cysts is consistent with the fact that KspCad-Cre is 100% penetrant in these tubule segments.\(^{35,38}\)
Ciliogenesis Is Inhibited in Cdc42 Kidney-Specific Knockout Mice

Given the ciliary phenotypes in cdc42AUG MO embryos (Figures 1–3), our data showing knockdown of Cdc42 in cell culture inhibits ciliogenesis,

18 and the fact that knockout of many ciliary proteins in mice leads to a polycystic phenotype,

2 we next investigated ciliogenesis in Cdc42 kidney-specific knockout mice. Immunostaining of kidney sections from control mice (Figure 7A) and Cdc42 kidney-specific knockout mice (Figure 7B) at P4 show a significant lack of cilia in the cystic areas of Cdc42 kidney-specific knockout mice but normal amounts of cilia in the unaffected tubules from these mice (Figure 7C). Scanning electron microscopy of kidneys from control (Figure 7D) and Cdc42 kidney-specific knockout mice (Figure 7E) at P6 also shows impaired ciliogenesis in the Cdc42 kidney-specific knockout mice. Cdc42 is involved in multiple cellular processes, such as polarity. In support of the idea that the phenotype here is mainly due to the effect of Cdc42 acting at the primary cilium in the cells surrounding cysts in the Cdc42 kidney-specific knockout mice, cell polarity appeared to be largely preserved, at least with respect to basolateral E-cadherin and apical gp135 localization (Supplemental Figure 1).

Increased Proliferation, Apoptosis, and Fibrosis in Cdc42 Kidney-Specific Knockout Mice

In many forms of PKD, especially nephronophthisis, levels of cell proliferation, apoptosis, and fibrosis are increased.

40 To investigate this, we first examined bromodeoxyuridine (BrdU) incorporation, a marker of active cell division, in sections of kidneys from the Cdc42 conditional knockout mice and wild-type littermate controls, and found it to be significantly increased in the Cdc42 conditional knockout kidneys (Figure 8, A–C). We next examined apoptosis, using terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining, and found it to be increased as well in Cdc42 kidney-specific knockout mice (Figure 8, D–F). Finally, we investigated fibrosis, using Masson trichrome staining, and it too was increased in the kidneys.
As a marker of increased fibrosis, we examined mRNA levels of collagen I and found them to be significantly increased in the kidneys of Cdc42 kidney-specific knockout mice (Figure 8I).

**MAPK Pathway Activation Occurs in Cdc42 Kidney-Specific Knockout Mice**

Finally, we examined a downstream signaling pathway, MAPK, that has been shown to be causally involved in PKD in the pcy nephronophthisis model. MAPK activation, specifically phosphorylation of the final protein ERK (pERK) in this pathway, was found to be significantly elevated by Western blot in three of five mice examined (Figure 9A). Importantly, in tissue sections, by immunohistochemistry, pERK was increased in kidney cyst cells in all five Cdc42 kidney-specific knockout mice examined (Figure 9B and Supplemental Figure 2). The fact that some cysts in the kidneys of these mice displayed minimal, or no, pERK activation (Supplemental Figure 2)
suggests that other signaling pathways (e.g., the planar cell polarity pathway, given the dilated tubules and disordered cilia seen in the cdc42AUG MO kidneys) might also be activated after Cdc42 knockout in kidney tubule cells.

DISCUSSION

A major strength of this study is that we used two different animal models, zebrafish and mouse, each of which has specific advantages that could be exploited to investigate the role of Cdc42 in renal ciliogenesis, cystogenesis, and regulation of the exocyst.

Zebrafish is a very useful animal model owing to the feasibility of studying genetic interactions, the ability to use antisense MOs for knockdown, the opportunity to quickly assay large numbers of embryos, and the ease of viewing organ phenotypes in living larvae. For these reasons, we initiated our Cdc42 studies in zebrafish.

We previously showed, in cell culture, that Cdc42 biochemically interacted with exocyst Sec10, co-localized with the exocyst at primary cilia, and that knockdown of Cdc42 and Tuba, a Cdc42 GEF, inhibited ciliogenesis and resulted in MAPK pathway activation. We show here that cdc42 knockdown in zebrafish phenocopies many aspects of sec10 and pkd2 knockdown, including tail curvature, glomerular expansion, and MAPK activation. Other ciliary phenotypes include hydrocephalus and loss of photoreceptor cell cilia. Indeed, we show ciliary mutant phenotypes in all the tissues (brain, eye, and kidney) in which cdc42 was highly expressed by in situ hybridization.

Using MOs to knock down cdc42 levels in vivo in zebrafish, we observe an absence of photoreceptor cell cilia, specifically the outer segments, in cdc42AUG MO embryos. We also saw hydrocephalus in cdc42AUG MO embryos, which can be due to defects in ependymal cell cilia in the ventricles of the brain. Although the absence of gross morphologic defects in pronephros cilia, and in cells surrounding the dilated ventricles of the brain, was initially surprising, it is possible that other isoforms of Cdc42—which are unaffected by our start-site

Figure 6. KspCre;Cdc42$^{fl/fl}$ mice develop renal cysts in the distal and collecting tubules. (A–D) Hematoxylin and eosin–stained sections of kidneys from control (A and C) and Cdc42 kidney-specific knockout mice (B and D) at P4 (A and B) and P6 (C and D). (E) Section of a control kidney incubated with LTA lectin, which stains the apical membrane of proximal tubule epithelial cells (brown color), and PNA lectin, which stains distal tubule and collecting tubule cells. (F and F') Section of a Cdc42 kidney-specific knockout kidney incubated with LTA lectin shows no staining of cysts (arrow in expanded inset to left), while a section incubated with PNA lectin shows staining of cysts (arrow in expanded inset to right), indicating that the cysts arise from distal tubules and collecting ducts. Bar for A and B, 200 μm. Bar for C–F, 20 μm.
MO—are sufficient to allow for cilia assembly in the kidney. Per the Zebrafish Model Organism Database, there are three zebrafish isoforms of human Cdc42, including isoform A (NP_956926), which is 98% identical, and is the sequence we used to generate the MO described here. Future studies will investigate the expression pattern of Cdc42 isoforms B and C, which might, for example, show expression in the kidney and in cells surrounding the ventricles of the brain, but not the eye. Nevertheless, these other isoforms were unable to restore complete pronephric cilia function during zebrafish development because cdc42 isoform A MO embryos still showed kidney cilia-related phenotypes that have been observed in other cilia mutants in zebrafish—such as edema, disordered cilia, and glomerular expansion. Therefore, we believe that the knockdown of the Cdc42 isoform most related to mammalian Cdc42, with a start-site MO, allowed us to uncover a role for cdc42 in pronephric cilia function. Maternal-zygotic cdc42 mutants of all three different isoforms, which could well result in early embryonic lethality, would likely be required to definitively say whether cdc42 is required for pronephric cilia formation in zebrafish. The inhibition of ciliogenesis that was seen in the cells surrounding cysts in Cdc42 kidney-specific knockout mice confirm the importance of Cdc42 for renal tubule cell cilia formation, and highlights the advantages of using multiple animal models to investigate these processes. Together with our previous work, our studies in zebrafish and mice demonstrate that Cdc42 is likely to be important for both kidney cilia formation and function.

We next took advantage of the robust ability to study genetic interactions in zebrafish. Using small amounts of cdc42 and sec10 MOs that alone generally had no effect, injection of both together resulted in a synergistic genetic interaction between cdc42 and sec10 for many of the cilia-related phenotypes. This suggests that cdc42 and sec10 act in the same pathway. We previously proposed that Sec10 and the exocyst are important for transporting proteins necessary for ciliary structure. Given that Cdc42 regulates the exocyst in yeast, we propose a model whereby Cdc42 is necessary for exocyst action at the primary cilium. If Cdc42 is required to regulate the exocyst, then MO co-injection would further impair ciliary

Figure 7. Primary ciliogenesis is inhibited in the cysts of Cdc42 kidney-specific knockout mice. (A and B) Immunostaining of acetylated α-tubulin (red) in kidneys from control (A) and Cdc42 kidney-specific knockout mice (B) at P4, shows lack of cilia in cells surrounding cysts. (C) Quantification from kidney tubule cell-specific Cdc42 knockout mice shows a significant decrease in the number of cilia seen per cell in cysts (which presumably are deficient in Cdc42 because no cysts were seen in control mice) compared with normal-appearing tubules, which most likely express Cdc42. Error Bars represent standard deviation and were calculated by Excel. (D) Scanning electron microscopy of kidneys from control (D, arrow) and Cdc42 kidney-specific knockout mice (E, arrow) at P6 shows abnormal-appearing, short, stubby cilia in cells surrounding cysts. Bar, 20 μm.
Sec10 levels beyond that seen after direct knockdown of Sec10 by a suboptimal dose of MO because a reduced amount of Sec10 would be present to act at the primary cilium.

Zebrafish have pronephric kidneys and mammals have metanephric kidneys; thus, we turned to mice to further investigate the role of Cdc42 in ciliogenesis and cystogenesis. Because global knockout of Cdc42 in mice results in early embryonic lethality, we used a floxed Cdc42 mouse created by the Brakebusch Group, and validated by that laboratory and others. We crossed these mice to kidney tubule cell-specific KspCad-Cre mice that had been successfully used to knock out other ciliary proteins, such as Kif3A, which also cause early embryonic lethality when knocked out globally.

The phenotype in our Cdc42 kidney-specific knockout mice, especially the multiple small cysts, increased cell proliferation, increased apoptosis, and increased fibrosis, is more consistent with a nephronophthisis model than ADPKD, in which greatly enlarged cystic kidneys are seen. Although less common than ADPKD, nephronophthisis, a group of autosomal recessive disorders, is the most frequent genetic cause of ESRD up to the third decade of life. Indeed, a recent study investigating the utility of whole exome sequencing in improving diagnosis and altering patient management found a mutation in an exocyst component, Exo84, in a family with Joubert syndrome.

**Figure 8.** Increase in cell proliferation, apoptosis, and fibrosis in kidneys of Cdc42 conditional knockout mice. (A and B) BrdU incorporation, a marker of active cell division, is significantly increased in sections of kidneys from the Cdc42 kidney-specific knockout mice (KO) compared with wild-type littermate controls. Bar, 10 μm. Quantification is shown in C. (D and E) Apoptosis, using TUNEL, was significantly increased in Cdc42 kidney-specific knockout mice compared with wild-type littermate controls. Quantification is shown in F. (G and H) Fibrosis, using Masson trichrome staining, was increased in the kidneys of Cdc42 kidney-specific knockout mice compared with controls. As a marker of increased fibrosis, we examined mRNA levels of collagen I and found them significantly increased in the kidneys of Cdc42 kidney-specific knockout mice (I). Bar, 10 μm. Error Bars in C, F, and I represent standard deviation and were calculated by Excel.
syndrome, a nephronophthisis-related disorder that also includes defects in brain (cerebellar) development. The p.E265G variant found in the family occurred in the B6 loop of the highly conserved pleckstrin homology domain, which is involved in binding phosphatidylinositol lipids for vesicular transport. This was the single, segregating variant in the family and was not present in 200 ethnically matched controls. It was predicted to be damaging by POLYPHEN-2,49,50 and occurred in a fully conserved residue.51 It should be noted, however, that we previously showed that polycystin-2, one of the two causative proteins in ADPKD, co-localized with the exocyst at the primary cilium18 and biochemically interacted with the exocyst.29 Cell proliferation is also a well known characteristic of ADPKD cells and plays a major role in the formation of the cysts that destroy the kidney, leading some to refer to ADPKD as “neoplasia in disguise.”52 Thus, involvement of Cdc42 and the exocyst in ADPKD is also quite possible.

Although we favor a model whereby the Cdc42 mutant phenotypes occur because of the central role of Cdc42 in ciliogenesis, we recognize the possibility that Cdc42 may play a different role because some proteins implicated in cilia function also have cilia-independent functions. Many ciliary proteins are not exclusively localized to the cilium, and it has recently been argued that polycystin function in the endoplasmic reticulum is more relevant for the observed curvy tail phenotype in zebrafish morphants.53 Indeed, multiple IFT proteins have recently been shown to have important functions in nonciliated cells.54

A key question, given the widespread localization of Cdc42 over the apical surface of renal tubule epithelial cells observed by us14 and others,39 then becomes, How is Cdc42 activated so that it can regulate the exocyst during ciliogenesis? A likely explanation is that one or more localized GEFs produce Cdc42-GTP activity at or near the primary cilium. Tuba, a Cdc42 GEF, was shown to be concentrated subapically, where the primary cilium forms.55 Knockdown of Tuba, in our previous study, inhibited ciliogenesis, although not to the same degree as knockdown of Cdc42.18 This may be due to incomplete knockdown of Tuba in our MDCK cells, or, alternatively, there may be one or more other GEFs that activate Cdc42 at the primary cilium. In a recent screen for modulators of ciliogenesis, 7784 therapeutically relevant genes across the human genome were tested using high-throughput small interfering RNA (siRNA).56 In that screen, intersectin 2 (ITSN2), another Cdc42 GEF, was shown to be a positive regulator of ciliogenesis. Importantly, ITSN2 was recently localized to the centrosome/basal body, which is at the base of the primary cilium, in MDCK cells.57 In the high-throughput siRNA screen by Kim et al., Cdc42 was listed as a rejected gene, based on “siRNA toxicity,” and the effect of Cdc42 knockdown on ciliogenesis was, therefore, not determined.56

Together, these findings provide the basis for a model in which the exocyst complex is localized to the primary cilium by activated Cdc42, is stabilized at the primary cilium by binding to the Par complex through Par6,18 and then targets and docks vesicles carrying proteins necessary for ciliogenesis (Figure 10). Given the importance of the primary cilium to many “ciliopathies,” including PKD, identifying the mechanisms of ciliary assembly governed by the exocyst could reveal novel therapeutic targets.

CONCISE METHODS

Ethics Statement
All zebrafish experiments were approved by the Institutional Animal Care and Use Committees at the University of Pennsylvania and the Philadelphia Veterans Affairs Medical Center. All mouse experiments were approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania.

Zebrafish Injections, MO Knockdown, and Rescue
Embryos were injected at the one-to-four-cell stage, and MOs were diluted with phenol red tracer (P0290, Sigma) at 0.05% and injected at 500 pl or 1 nl per embryo. cdc42AUG and sec10e2i2 MOs, designed against zebrafish cdc42 and sec10, were purchased from Gene Tools, LLC: cdc42AUG-MO (5’-CAACGACGACTTGATCGTCTGCAT-3’), sec10e2i2-MO (5’- AAATTTCTGTAACTCATTCTTATTGG-3’). cdc42AUG-MO was designed to target the ATG start codon. sec10e2i2-MO was described previously.29 MOs were injected as single doses of 1, 2, or 3 ng cdc42-MO (designated in the text as cdc42AUG MO) or a combined dose of 1 or 2 ng cdc42AUG MO plus 7.5 ng sec10e2i2-MO (designated in the text as 1 or 2 ng cdc42AUG MO plus 7.5 ng sec10 MO) per embryo. Capped mouse Cdc42 full-length mRNA was synthesized using the mMessage mMACHINE T7 kit as instructed by the manufacturer (AM1344, Ambion). For the rescue
experiments, 10–30 pg of Cdc42 mRNA was co-injected with the MOs into one-to-four–cell stage embryos.

Whole Mount In Situ Hybridization and Immunofluorescence in Zebrafish

Total RNA was isolated from 48 hpf zebrafish embryos using the RNAeasy Plus mini kit (74134, Qiagen) according to the manufacturer’s instructions. cDNA was synthesized using the high-capacity cDNA reverse transcription kit (4368814, Applied Biosystems) as described in the manual. To detect the cdc42 expression pattern in vivo, RNA probes were synthesized as previously described.58,59 The primer sequences for RNA probes are listed as follows (the T7 promoter sequence is highlighted in bold): Antisense (forward: 5'-GGGTTGTTGGTTGATGTC-3', reverse: 5'-TAATAGCAGTCGTATAGGG-3'), sense (forward: 5'-TAATACGACTCACTATAGGG-3'), reverse: R: 5'-AGCCTTCAGATCGCGGGCCA-3'). DIG-labeled RNA probes were used for RNA in situ hybridization using standard methods.50 The wula probes were prepared as previously published.29 Immunofluorescence for cilia staining in zebrafish was performed similar to previously published protocols.61

Quantification of Glomerular Size

Glomerular width was measured with the Fiji (Image J) software, version 1.47g, on images of whole embryos collected using a Leica M205 C microscope and a DFC450 digital camera. Glomerular width was evaluated by collecting one image at the area of widest diameter from each mutant or wild-type embryo.

Histology

Zebrafish and mouse tissues were fixed in 4% paraformaldehyde overnight at 4°C. After gradual dehydration into ethanol, tissues were embedded in paraffin, and sectioned at 4- to 5-μm thickness. Some of these sections were stained in hematoxylin and eosin.

Western Blot Analysis

Zebrafish embryo lysates were prepared with 3–5 dpf embryos as previously described.29 Mouse kidney lysates were homogenized in
RIPA buffer (R0278, Sigma) containing a protease inhibitor cocktail (P2714, Sigma) and phosphatase inhibitor (78420, Thermo), and the lysates were centrifuged at 13,500 rpm for 20 minutes at 4°C. Supernatants were collected and protein concentration was determined using the bicinchoninic acid protein assay (23227, Thermo). The protein samples were separated on NuPage 4–12% Bis-Tris gels (NP0336, Novex) and then transferred to a Nitrocellulose Membrane (LC2000, Novex).

Antibodies
The antibodies used in this study were mouse monoclonal anti-Cdc42 (610929, BD Transduction Laboratories), rabbit polyclonal anti-phospho-ERK1/2 (#9101, Cell Signaling), rabbit polyclonal anti-total ERK1/2 (#9102, Cell Signaling), mouse monoclonal anti–glyceraldehyde 3-phosphate dehydrogenase (G8795, Sigma), rabbit polyclonal anti-β-tubulin (T5192, Sigma), mouse monoclonal antiacetylated α-tubulin (T6793, Sigma), rat monoclonal anti-Brdu (ab6326, Abcam), mouse anti-GP135 (a gift from Dr. George Ojakian, State University of New York), rabbit anti-E-cadherin (ab15148, Abcam), biotinylated LTA (NP0336, Novex) and then transferred to a Nitrocellulose Membrane (BASIC RESEARCH 1435–1450, 2013) using the VECTASTAIN Elite ABC kit (PK-6101 and PK-6102, Vector Laboratories) and counterstained in hematoxylin. For the immunofluorescence staining, the 4% paraformaldehyde-fixed, paraffin-embedded sections were dewaxed, rehydrated in graded ethanol, and retrieved with 10 mM sodium citrate (pH, 6.0). Sections were blocked in PBS containing 5% donkey serum and then incubated with antibodies.

Proliferation and Apoptosis Assays
Proliferation was assayed using BrdU incorporation. Two hours before harvesting of the kidneys, mice received intraperitoneal injections of 100 mg/kg body weight BrdU solution (B9285, Sigma-Aldrich). Apoptosis was determined using ApopTag Peroxidase In Situ Apoptosis Detection Kit (S7100, Chemicon) as directed by the manufacturer’s instructions. For BrdU and apoptosis (TUNEL) staining, positive nuclei were counted per high-power field (600×) in both control and knockout kidneys, and the percentage positivity was calculated per area.

BUN Analyses to Measure Kidney Function
Serum was prepared as described previously.63 Briefly, serum was separated from blood collected from the 6-, 10-, and 33-day-old mice and was stored at −80°C for further analysis. BUN levels were quantified from serum (analyzed by the Ryan Veterinary Hospital Laboratory, University of Pennsylvania).

Electron Microscopy
Kidneys from Cdc42 kidney-specific knockout mice and their littermate controls were bisected longitudinally after removal and were fixed in a solution containing 2% glutaraldehyde, 0.8% paraformaldehyde, and 0.1 M cacodylate. The fixed tissue was sectioned and rinsed with 100 mM of cacodylate buffer, dehydrated through a graded ethanol series, washed with hexamethyldisilazane (Electron Microscopy Sciences), coated with platinum after air-drying for 5 minutes at 60°C, and analyzed on a scanning electron microscopy machine (XL20 SEM, Phillips Inc.).

Imaging
All images were captured in TIF format and processed in Adobe Photoshop CS5.1. For immunofluorescence, zebrafish embryos and mouse tissue were imaged on an Olympus BX42 and a Zeiss Axio Observer D1m. For histology and in situ hybridization, samples were imaged using a Leica M205C Light Microscope.

Statistical Analyses
Gliomerular size was compared using the Wilcoxon rank-sum test. Phenotypes for the epistasis experiments were classified as abnormal, after MO injection, by the presence of any combination of the following features: edema, short body size, hydrocephalus, and small eyes. Logistic regression and chi-squared testing were used to compare the proportion of abnormal phenotypes across groups. Confidence intervals and P values were adjusted for within-trial correlation by clustering at the trial level, and robust SEM estimates were generated.
These tests were performed using Stata software, v12.1 (Stata Corp., College Station, TX). For comparison of means, a t test was performed using SPSS software (v.15.0) (SPSS Inc., Chicago, IL). For all tests, \( P < 0.05 \) was considered to represent statistically significant differences.

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DISCLOSURES

None.

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