Published in final edited form as:

Traffic. 2012 September ; 13(9): 1295–1305. doi:10.1111/j.1600-0854.2012.01387.x.

OCRL1 Modulates Cilia Length in Renal Epithelial Cells

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Abstract

Lowe syndrome is an X-linked disorder characterized by cataracts at birth, mental retardation and progressive renal malfunction that results from loss of function of the OCRL1 (oculocerebrorenal syndrome of Lowe) protein. OCRL1 is a lipid phosphatase that converts phosphatidylinositol 4,5-bisphosphate to phosphatidylinositol 4-phosphate. The renal pathogenesis of Lowe syndrome patients has been suggested to result from alterations in membrane trafficking, but this cannot fully explain the disease progression. We found that knockdown of OCRL1 in zebrafish caused developmental defects consistent with disruption of ciliary function, including body axis curvature, pericardial edema, hydrocephaly and impaired renal clearance. In addition, cilia in the proximal tubule of the zebrafish pronephric kidney were longer in *ocr1* morphant embryos. We also found that knockdown of OCRL1 in polarized renal epithelial cells caused elongation of the primary cilium and disrupted formation of cysts in three-dimensional cultures. Calcium release in response to ATP was blunted in OCRL1 knockdown cells, suggesting changes in signaling that could lead to altered cell function. Our results suggest a new role for OCRL1 in renal epithelial cell function that could contribute to the pathogenesis of Lowe syndrome.

Keywords

kidney; Lowe syndrome; MDCK; oculocerebrorenal syndrome of Lowe; phosphatidylinositol 4,5-bisphosphate; zebrafish

Lowe syndrome is an X-linked disorder characterized by cataracts at birth, mental retardation and renal malfunction that results from mutations in the gene encoding oculocerebrorenal syndrome of Lowe (OCRL1) (1). OCRL1 is a lipid phosphatase that

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hydrolyzes the 5' phosphate of phosphatidylinositol 4,5-bisphosphate (PIP₂) and phosphatidylinositol 3,4,5-trisphosphate (PIP₃), with a preference for the former substrate *in vitro* (2,3). OCRL1 is localized primarily at the *trans*-Golgi network (TGN), but is also present on a subset of endosomes and associated with clathrin-coated pits (4–8). Mutations that cause Lowe syndrome result in complete loss of OCRL1 catalytic activity (2,9). OCRL1 appears to be the major PIP₂-hydrolyzing enzyme in human renal proximal tubule cells, and cells lacking OCRL1 have slightly elevated levels of PIP₂ (3,10).

Lowe syndrome patients almost universally present with renal disease from birth, including acidosis, amino aciduria, phosphaturia and low molecular weight proteinuria (11). The defect in protein reabsorption may result from improper function or trafficking of the cell surface protein megalin, a large multiligand receptor that recycles at the apical domain of polarized epithelial cells (12). The demonstration that a subpopulation of OCRL1 binds to the endosomal adaptor APPL1 in clathrin-coated pits has fueled this speculation (6). OCRL1 also interacts with clathrin, α -adaptin and numerous Rab proteins, including Rab5 and Rab6, as well as Ses/IPIPs (4,8,13–16).

The potential link between loss of OCRL1 function and defective megalin trafficking has been reinforced by studies of Dent disease, originally described as resulting from defective function of the CLC-5 Cl⁻/H⁺ antiporter. Dent disease patients present with renal symptoms that are similar, though not identical to Lowe syndrome patients; however, Dent disease patients have none of the extra-renal symptoms characteristic of Lowe syndrome. There is strong evidence that loss of CLC-5 function leads to decreased uptake of fluid phase markers and megalin ligands in knockout mouse models (17–22). Surprisingly, a significant fraction of patients diagnosed with Dent disease were recently found to have mutations in OCRL1 rather than CLC-5; these patients are now classified as having Dent 2 (23). Mutations in OCRL1 that cause Dent 2 are limited to nonsense or splicing mutations within the first seven exons of the gene or to missense mutations in the catalytic domain. It is likely that these mutations result in expression of alternatively spliced or partially active variants of OCRL1 that are sufficient for function in extrarenal tissues, but not in the kidney (24).

In disagreement with the prevailing hypothesis for OCRL1 function in the kidney, we found that endocytic trafficking of megalin and a megalin ligand was unaffected by knockdown of OCRL1 in polarized canine and human renal epithelial cells (10). More recent studies have suggested that OCRL1 depletion alters endocytic recycling, possibly by modulating actin dynamics, in nonpolarized cells (14,25). The varying results obtained using these models could reflect the fact that apical endocytosis is considerably slower than internalization in nonpolarized cells and is more difficult to quantitate, or might suggest a differing role for actin dynamics in these processes. A common finding by several groups is that loss of OCRL1 results in altered trafficking of the mannose 6-phosphate receptor leading to enhanced secretion of lysosomal hydrolases (4,10,25). This finding is consistent with clinical observations that Lowe syndrome patients have elevated plasma levels of lysosomal hydrolases (4,26).

Given the lack of a clear effect of OCRL1 knockdown on megalin trafficking in polarized cells, we have revisited the question of how loss of OCRL1 disrupts organ function in Lowe syndrome patients. Interestingly, while OCRL1 is ubiquitously expressed in tissues, the organs most affected in Lowe syndrome (eye, brain and kidney) are also affected in diseases caused by disruption of ciliary function (collectively termed ciliopathies). Several features of OCRL1 are also consistent with a potential role in regulating ciliary biogenesis. The ASH domain in OCRL1 has been found in several proteins localized to cilia as well as to the Golgi complex (27). In addition to its selectivity for PI4,5P₂, OCRL1 is able *in vitro* to hydrolyze PI3,4,5P₃ to PI3,4P₂, and the generation of this product may play an important

role in delivery of cargo to cilia (28). Moreover, OCRL1 is known to bind Rab8, a protein implicated in ciliary biogenesis (16,29–31). Also of note is that another PIP₂ phosphatase, INPP5E, has been implicated in the ciliopathy MORM syndrome (32). With this in mind, we asked whether OCRL1 might play a role in ciliary biogenesis or function.

In our studies, we found that knockdown of OCRL1 in zebrafish disrupted renal function and resulted in phenotypes consistent with ciliary defects. Moreover, we observed that primary cilia of Madin-Darby canine kidney (MDCK) cells depleted of OCRL1 were significantly longer than those of control cells. Similar to cells depleted of galectin-3, which also have elongated cilia, we found that MDCK cells grown in three-dimensional cultures formed aberrant cysts when OCRL1 was depleted. Additionally, cell signaling is apparently aberrant in OCRL1 depleted cells, as the intracellular release of calcium in response to ATP challenge was reduced. Our results suggest that loss of OCRL1 function affects multiple cellular processes that contribute to disease pathogenesis in the kidney.

Results

Knockdown of OCRL in zebrafish disrupts embryonic development and renal clearance

Knockout of the OCRL1 gene in mice does not recapitulate Lowe syndrome, likely due to expression of a homologous inositol polyphosphate-5-phosphatase called INPP5B. As an alternative approach to assess the role of OCRL1 in renal function, we examined the expression and function of *ocrl* in the zebrafish *Danio rerio*. Zebrafish have been widely used to model human disease, including many ciliopathies that disrupt kidney development and function (33,34). Zebrafish express a single copy of *ocrl* that is 59% identical at the amino acid level to human OCRL1, with the greatest identity found within the catalytic and RhoGAP domains of the protein. There is one INPP5B homolog in zebrafish (69). RT-PCR using primers specific for zebrafish *ocrl* revealed expression of *ocrl* mRNA in embryo extracts as early as the 13-somite stage and persisting for at least 48 hours postfertilization (hpf) (Figure 1A). *In situ* hybridization analysis using an antisense probe confirmed *ocrl* mRNA expression in the pronephric kidney, brain and eye at early stages and persisting through 48 hpf (Figure 1B, C). No expression was observed using a sense probe at 24 and 48 hpf (Figure 1D).

We next assessed the effect of knocking down *ocrl* using a translation-blocking morpholino (MO). The *ocrl*-depleted embryos were developmentally impaired, and exhibited abnormal body curvature, hydrocephaly and pericardial edema. Morphants were classified at 48 hpf as wild type (class I), moderate (class II) and severe (or dead, class III) based on the extent of body curvature and edema (Figure 2A). The percentage of moderately and severely affected morphants increased with the dose of *ocrl*/MO injected, with 6, 6.5 and 7 ng MO yielding approximately 70, 80 and 100% class II and class III phenotypes, respectively (Figure 2B).

To confirm that the effects we observed were due to loss of *ocrl*, we attempted to rescue the phenotypes by expressing MO-resistant *ocrl* mRNA. Embryos were injected at the one-cell stage with 200 pg of synthetic wild-type zebrafish *ocrl* mRNA followed by injection of 6 ng *ocrl* or control MO before the eight cell stage. Phenotypes were then analyzed at 48 hpf. Injection of 200 pg *ocrl* mRNA without subsequent MO injection had no deleterious consequences on embryonic development (not shown). Importantly, expression of wild-type *ocrl* partially rescued the morphant phenotype, with only approximately 40% of embryos exhibiting moderate (class II) or severe (class III) phenotypes (compared with 70% induced by injection of 6 ng *ocrl*/MO alone). In contrast, injection of the *ocrl*/R559G, which contains a mutation in the conserved catalytic domain found in Lowe syndrome patients, failed to rescue the morphant phenotype (Figure 2B).

Notably, the defects in embryonic development that we observed are consistent with compromised kidney and ciliary function in zebrafish. We therefore asked whether renal function was impaired in *ocr1* morphant embryos. Morphant and control embryos were injected in the common cardinal vein with 10 kDa rhodamine dextran at 48 hpf and renal clearance was followed over time using fluorescence imaging. Only moderately affected morphants (class II) were selected for these experiments to minimize indirect effects due to severe developmental defects. Whereas embryos injected with the control MO efficiently cleared the dextran over a 24-h period, embryos injected with the *ocr1* MO retained significant amounts of dextran in the circulatory system, consistent with aberrant fluid clearance (Figure 3). The impaired clearance was not due to blockage of the pronephric duct, as there was no obstruction of the cloaca in these morphants (Figure S1).

The pronephric kidney tubule contains both monociliated and multiciliated cells (35,36). To determine whether knockdown of *ocr1* altered cilia morphology, we visualized cilia in the pronephric kidney tubule at 24 hpf using anti-acetylated tubulin. We also included antibodies against IFT88, which localizes in part to basal bodies (37). We consistently observed that the cilia were more disorganized in the morphants, consistent with a widening of the tubule (Figure 4). We also quantified the length of the cilia in the pronephric kidney. The average length of cilia was 5.2 μm in embryos injected with control MO. Interestingly, cilia were elongated in *ocr1* morphants (average length of 6.7 μm ; Figure 4). We also imaged cilia in the Kupffer's vesicle at the 13-somite stage and found no difference in cilia abundance or length between morphants and controls (data not shown).

Knockdown of OCRL1 in renal epithelial cells results in elongation of cilia

The phenotypes and defects in pronephric kidney function of zebrafish morphants led us to examine the effect of OCRL1 knockdown on cilia morphology and function in mammalian renal epithelial cells. To this end, MDCK cells were transfected with control siRNA or siRNA targeting OCRL1, cultured for 4 days on permeable supports, and processed for indirect immunofluorescence with anti-acetylated tubulin to visualize and quantitate cilia length. Western blotting confirmed that knockdown of OCRL1 was efficient (typically >80%, Figure 5A). Interestingly, we observed that cilia on cells transfected with OCRL1 siRNA were clearly longer than cilia on cells treated with control siRNA (Figure 5B). Cilia lengths from 100 cells for each condition were measured using VOLOCITY software, sorted by length, and plotted in rank order from shortest to longest (Figure 5C). Typically, approximately 90% of cilia were <5 μm in length in control cells, although a few very long cilia (up to ~20 μm) were also seen. In OCRL1 depleted cells, the shortest cilia observed were the same length as control, but the distribution of lengths rapidly diverged. Rank sum analysis confirmed that the median length (3.38 μm versus 1.72 μm for cells treated with OCRL1 versus control siRNA, respectively) was statistically different ($p < 0.001$) in cells treated with OCRL1 siRNA compared with control. This observation was extremely reproducible: cilia lengths in OCRL1-depleted cells were markedly longer than control in 19 of 23 experiments, and in no experiment was the mean cilia length in cells treated with OCRL1 siRNA shorter than control.

In the course of our studies, we used several different sub-clones of MDCK cells and noted some variation in mean cilia length between these. However, in all cases, depletion of OCRL1 resulted in elongation of cilia compared with cells treated with control siRNA (data not shown). Additionally, when differentiated, cilia-bearing cells were acutely deciliated using ammonium sulfate and then allowed to recover for 2 days, the restored cilia in OCRL1-depleted cells were again longer than control (Figure S2).

Loss of function of INPP5E, a phosphatidylinositol 5'-phosphatase that localizes to cilia, has been shown to cause MORM syndrome, a cystic disease in which cilia are characteristically

sparser than normal and have dilated ends (32). We therefore compared cilia morphology in control and OCRL1-depleted cells using scanning electron microscopy. While there was some variability in cilia length within each population, we found no obvious morphological differences between control and knockdown cells (Figure 5D).

We attempted to rescue the effect of OCRL1 knockdown on cilia length by heterologous expression of GFP-tagged siRNA-resistant wild type and mutant OCRL1. For these experiments, we generated stable cell lines expressing GFP-tagged wild type and catalytically inactive siRNA-resistant OCRL1 constructs, transfected the stably expressing and parental lines with control or OCRL1 siRNA and then quantitated cilia length in GFP-positive cells. These experiments proved difficult to interpret with confidence, as the stable cell lines that we isolated had variable cilia lengths compared with the parental controls. It is possible that overexpression of OCRL1 contributed to the variability in length and to the extent of rescue that we observed. Ultimately, these disparities made it difficult to rigorously assess whether overexpression of OCRL1 restored cilia length to control levels.

Although OCRL1 is ubiquitously expressed in tissues, many organs and cell types retain normal function in Lowe syndrome patients. We therefore asked whether OCRL1 depletion modulates cilia length in fibroblasts. We obtained essentially complete knockdown of OCRL1 in these cells (Figure 6A); however, this did not alter the cilia length profile of these cells measured after 3 days of serum starvation (Figure 6B) or under-fed conditions (data not shown). Upon shorter starvation periods, cilia in OCRL1-depleted fibroblasts were slightly longer than controls, consistent with our data in kidney cells (Figure 6C).

Knockdown of OCRL1 disrupts lumen formation in cyst cultures

Increased cilia length has been linked with defective growth of MDCK cells in three-dimensional cyst cultures (38). We therefore examined the effect of OCRL1 knockdown on cilia and morphology of MDCK cysts. Cells transfected with control or OCRL1-specific siRNA were plated in Matrigel and fixed after 5 days in culture. Cells were processed for indirect immunofluorescence with anti-acetylated tubulin and anti-ZO-1 antibodies and cilia length was quantitated. As a positive control, we knocked down galectin-3, which has previously been demonstrated to result in elongated cilia (38). Cilia in cyst cultures of OCRL1-depleted cells were visibly longer compared with control, and quantitation confirmed that knockdown of either OCRL1 or galectin-3 caused elongation of cilia (Figure 7A, B). Moreover, we observed that there was a greater fraction of abnormal cysts (with either multiple lumens or filled lumen) in both OCRL1 and galectin-3 depleted cultures (Figure 7C). Interestingly, another recently published study has reported a similar effect of OCRL1 knockdown on cyst morphology (39).

Calcium signaling in OCRL1-depleted cells

Mutations that result in shortened primary cilia are known to cause defects in cellular ATP release and calcium signaling that are thought to contribute to renal cyst formation (40–43). However, the effect of longer cilia on calcium signaling has not been examined. MDCK cells treated with control or OCRL1 siRNA were cultured on coverslips and loaded with Fura-2. Cells were then subjected to fluid shear and intracellular calcium was measured using ratiometric imaging. We did not detect any difference in calcium mobilization upon initiation of fluid shear in cells treated with control versus OCRL1 siRNA (data not shown). However, calcium mobilization in response to addition of extracellular ATP was significantly reduced in OCRL1-deficient cells compared with controls (Figure 8).

Discussion

In the studies reported above we found that knockdown of OCRL1 in zebrafish elicited phenotypes consistent with a defect in cilia function and compromised pronephric kidney function. Additionally, loss of OCRL1 caused elongation of primary cilia in the zebrafish pronephric kidney as well as in mammalian renal epithelial cells and disrupted cyst formation in three-dimensional cell cultures. Calcium signaling in response to ATP challenge was also aberrant in renal cells depleted of OCRL1. Together, these results suggest that in addition to the known effects of OCRL1 depletion on membrane traffic and actin dynamics, altered ciliary biogenesis may also contribute to the pathogenesis of Lowe syndrome.

Indeed, notwithstanding the initial focus on a putative role of OCRL1 in modulating endocytosis, several recent studies have observed more global functions for this protein in cell polarity and division. Impaired cell migration and spreading have been demonstrated in Lowe syndrome patient cells (44). More recently, Grieve et al. (39) reported a dramatic effect of OCRL1 knockdown on renal cyst morphology and cell proliferation. Additionally, this study found that OCRL1 localized in part to tight junctions. Two other recent reports have implicated OCRL1 in actin and lipid dynamics required for cytokinesis (45,46). Given the relationship between cell division, actin dynamics and ciliogenesis (47,48), it is tempting to speculate that the effect we observed on ciliary length is related to this function of OCRL1. How the role of OCRL1 in cytokinesis contributes to the phenotypic defects in Lowe syndrome remains unclear.

Could Lowe syndrome be a ciliopathy?

The link between OCRL1 and cilia function suggests the possibility that Lowe syndrome might constitute a novel class of ciliopathy. While the analogy is certainly not perfect, there are interesting comparisons to make. Whereas all defects that lead to shortened cilia in renal cells are believed to result in cystic disease, some reported ciliopathy mutations instead cause elongation of cilia (49–51). Elongated cilia have also been observed in mouse renal tubules during the recovery from acute kidney injury and ureteral obstruction (52,53). Although ciliopathies frequently affect the same organs compromised in Lowe syndrome, many of the clinical features differ significantly. Patients with known ciliopathies do not typically have proteinuria, and instead present with cystic kidney disease and occasional *situs inversus*, neither of which is observed in Lowe syndrome. Moreover, while ocular problems in ciliopathy patients result from retinal degeneration, Lowe syndrome patients are born with cataracts resulting from necrosis of the lens epithelium. That said, many of the features commonly observed in Lowe syndrome, including cataracts, hypotonia and polyuria, high pitched voice and deep set eyes, have been reported in patients with Bardet-Biedl syndrome (BBS) (54). BBS patients can have mutations in any of the 12 protein subunits that comprise the BBSome, a complex that regulates cargo delivery to cilia, and some BBS knockout mice have elongated cilia (49). Moreover, similar to the variation in symptoms observed between mutations in OCRL1 that cause Lowe syndrome versus Dent 2 disease, mutations in the same ciliary gene can result in very different syndromes (54). For example, different mutations in the nephrocystin-3 protein that cause nephronophthisis can variably result in cystic kidney disease only, or in the broad range of clinical symptoms that include polydactyly, *situs inversus* and heart defects (55).

A new working model for OCRL1 function

On the basis of our own studies and the published work by others, we propose important functions for OCRL1 in both TGN and endosomal trafficking steps. First, localized OCRL1-mediated cleavage of PIP₂ in the TGN and/or endosomes to produce phosphatidylinositol 4-

phosphate (PI4P) is important for the efficient delivery of newly synthesized lysosomal hydrolases. These enzymes bind to the mannose 6-phosphate receptor (M6P-R) in the Golgi/TGN, and are ferried to early and ultimately late endosomes, where dissociation occurs. Subsequently, transient fusion of late endosomes with lysosomes enables delivery of the soluble hydrolases and recycling of the M6P-R. Importantly, the adaptor proteins that bind to M6P-R, including epsin R, AP-1, and the GGAs, all bind to phosphatidylinositol 4-phosphate (PI4P). While the abundant source of PI4P generated in the Golgi complex by phosphatidylinositol 4-kinases has been assumed to supply this lipid, the observations that lysosomal hydrolases are preferentially secreted and that M6P-R trafficking is disrupted in cells lacking OCRL1 suggests that this enzyme may also generate a functionally important pool of PI4P along the pathway.

How might OCRL1 modulate cilia length and how might this contribute to the kidney and/or extrarenal symptoms observed in Lowe syndrome? One possibility is that OCRL1 functions to generate a pool of PIP₂ required for maintaining ciliary transport. Phosphoinositides are known to play roles in protein delivery to cilia (56,57). An intriguing target for these lipids is the exocyst, which plays a role in ciliary biogenesis and is known to bind to PIP₂ (58–60). Alternatively, OCRL1 is able *in vitro* to hydrolyze PI3,4,5P₃ to PI3,4P₂, and the generation of this product may play an important role in delivery of cargo to cilia (28,61). Recent studies by Nachury and colleagues have found that the BBSome, a coat-forming complex that together with Rab8 directs transport carriers to cilia (28,31), binds selectively to PI3,4P₂ (28). OCRL1 function in endocytic compartments might contribute to the pool of PI3,4P₂ recognized by the BBSome. Notably, OCRL1 also interacts with Rab8 (16). While a detailed mechanism remains elusive, loss of OCRL1 might enhance delivery of some ciliary proteins by altering the localization or activity of the BBSome. The resulting elongated cilia may respond aberrantly to flow or other signaling cues in the renal tubule, resulting in dysfunctional downstream responses that could contribute to the renal pathology observed in Lowe syndrome patients.

While this manuscript was in preparation, two reports appeared in press that also document effects of OCRL1 depletion on cilia in zebrafish and in mammalian fibroblasts (62,63). Surprisingly, knockdown of OCRL1 in fibroblasts and retinal pigment epithelial cells was found to decrease cilia length in these studies. Although neither study examined cilia in renal cells, reduced cilia length in OCRL1-depleted cells is a surprising observation given the strong link between shortened cilia and formation of renal cysts, which are not a feature of Lowe syndrome. Clearly, additional experiments will be necessary to reconcile the disparate observations and to refine our understanding of the role of OCRL1 in ciliary biogenesis and function.

Materials and Methods

Zebrafish husbandry

Zebrafish were maintained under standard conditions and staged as described previously (64). Embryos were collected from group matings of wild-type adult zebrafish. All animal husbandry adheres to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

In situ hybridization

In situ hybridization was performed as previously described (64). *ocrl* full-length cDNA was purchased from Open Biosystems and the *ocrl* probe was made using the DIG labeling kit (Roche). T7 RNA polymerase was used for RNA synthesis.

Real-time PCR

Zebrafish embryos were homogenized with a plastic microcentrifuge pestle in 500 μ L of TRI reagent (Ambion), and RNA was isolated using the RNeasy Micro Kit (Qiagen) according to the manufacturer's protocol. Primer sets against *ocrl* were designed using PrimerQuest on the IDT Web site (forward primer: 5'-AGA CCA GTG ACC ACA AAC CTG TCA-3'; reverse primer: 5'-AGA ATG TCA TCC GGG CTT TCT CCA-3'). Synthesis of cDNA was performed using M-MLV reverse transcriptase (Ambion) according to the manufacturer's recommendations. PCR was performed using the BioRad icycler and GeneAmp[®] High Fidelity PCR System (Applied Biosystems). The denaturing temperature was 95°C, the annealing temperature was 55°C and the extension temperature was 68°C with an amplification cycle of 30 min.

Microinjection

Zebrafish embryos were injected up to the eight-cell stage with the indicated doses of a translation blocking MO targeting *ocrl* (CGGAAATCCCAAATGAAGGTTCCAT) or a standard control MO. The same sequence targeting *ocrl* was recently also validated by Coon et al. (62). Embryos were allowed to develop in E3 culture medium (5 mM NaCl, 0.17 mM KCl, 0.4 mM CaCl₂, 0.16 mM MgSO₄) at 28.5°C. Images were taken and phenotypes classified at 48 hpf. For rescue experiments, MO-resistant wild type and R559G OCRL were prepared using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) and mRNAs were synthesized using mMESSAGE mMACHINE[®] SP6 kit (Invitrogen) according to the manufacturer's recommendation. Zebrafish embryos were injected at the one-cell stage with 200 pg of synthetic wild type or mutated *ocrl* mRNA, and embryos were imaged and classified at 48 hpf.

Rhodamine dextran clearance assay

Renal function was assayed as previously described (65,66). Briefly, 1 ng of 10 kDa Rhodamine dextran was injected into the common cardinal vein of embryos at 48 hpf. Embryos were imaged sequentially and under identical conditions at 1, 5 and 24 h post-injection. The loss of fluorescence near the common cardinal vein area over time was quantified using Adobe Photoshop.

Indirect immunofluorescence of cilia in zebrafish

Embryos at 48 hpf were incubated in Dent's fixative (80% methanol and 20% DMSO) overnight at 4°C. After rehydration, embryos were incubated with block solution (PBS with 0.5% Tween 20 with 10% Normal Goat Serum) for 1 h at room temperature and then overnight at 4°C with mouse anti-acetylated tubulin (1:1000, 6-11B-1, Sigma) and rabbit anti-IFT88 (1:5000, gift from Brian Perkins; reviewed in 67). Alexa Fluor 568 goat anti-mouse (1:1000, Invitrogen) and Alexa Fluor 488 goat anti-rabbit (1:1000, Invitrogen) were used as secondary antibodies. Images of kidney tubules were acquired using a Leica DMI 6000 CS Trino microscope and processed and quantified using ImageJ.

Cell culture, siRNA knockdown and generation of stable cell lines

Type II MDCK cells were transfected with siRNA oligonucleotides targeting OCRL1 (10), galectin-3 (68) or luciferase (as a control) prior to plating on filter supports as described in Mo et al. (68) and used for experiments 4 days later. Duplicate filters were processed for western blotting with monoclonal anti-OCRL1 antibody (gift of Robert Nussbaum and Sharon Suchy) to monitor the efficiency of knockdown. Human fibroblast cultures were transfected with OCRL1 or control siRNA and incubated in serum-free medium the following day. Cells were processed for western blotting or indirect immunofluorescence 4 days after transfection.

Immunofluorescence and quantitation of cilia length

Filter- or coverslip-grown cells were fixed with 4% paraformaldehyde in 100 mM sodium cacodylate, 3 mM CaCl_2 , 3 mM MgCl_2 and 3 mM KCl, pH 7.4 for 15 min (5 min at 37°C followed by 10 min at ambient temperature), quenched in PBS with 20 mM glycine and 75 mM NH_4Cl for 5 min, permeabilized using 0.1% TritonX-100 in the quench solution for 10 min with gentle shaking, blocked in PBS with 1% fish skin gelatin (Sigma) and 0.1% saponin for 10 min at 37°C. This was followed by sequential incubation for 1 h with mouse monoclonal anti-acetylated tubulin antibody (1:400; Sigma) and fluorophore-coupled goat anti-mouse secondary antibody (1:500; Invitrogen) diluted in PBS with 0.5% gelatin and 0.025% saponin. After thorough washes between and after antibody incubations, squares of transwell filters (cut out from the transwells) or coverslips were mounted on glass slides using Prolong Gold antifade reagent with DAPI (Invitrogen). Mounted slides were dried overnight at room temperature. Random fields of cilia were acquired using a Leica DM6000B upright fluorescence microscope. Individual cilia lengths (at least 100 per sample) were measured using VOLOCITY software, sorted in ascending order by length and plotted from shortest to longest.

Scanning electron microscopy

MDCK cells were transfected with siRNA, plated on filters for 5 days, then fixed in 200 mM sodium cacodylate buffer, pH 7.4 containing 0.5% glutaraldehyde, 2% paraformaldehyde, 0.5 mM MgCl_2 and 1 mM CaCl_2 for 30–60 min at ambient temperature. Filters were washed with 100 mM sodium cacodylate buffer, pH 7.4, for 5 min. Subsequently, cells were fixed again with 1.5% OsO_4 in 100 mM sodium cacodylate buffer, pH 7.4, for 1 h at 4°C with gentle shaking in a fume hood, rinsed repeatedly with deionized water, and washed for 5 min in deionized water with gentle shaking. Following fixation, cells were dehydrated by sequential soaking for 5 min each on ice in 40, 50, 75, 80, 90 and 95% ethanol. Cells were then incubated twice in 100% ethanol for 10 min at ambient temperature, critical point dried on a Samdri® -PVT-3D machine (Tousimis), and sputter coated using gold palladium in a Cressington Sputter Coater. Samples were imaged using a JEOL JSM-T300 scanning electron microscope.

Culture and staining of MDCK cysts

MDCK cells were detached from culture plates using trypsin and diluted to a final concentration of 2.5×10^4 cells/mL. Aliquots (0.2 mL) were pelleted, resuspended in 50 μL Matrigel Basement Membrane Matrix (BD Biosciences) and plated on cover slips. The solution was allowed to solidify at 37°C in 5% CO_2 for 30 min prior to addition of growth medium. The cells were cultured and fed every 24–48 h for 5–7 days.

For immunofluorescence staining, cysts were fixed in 4% paraformaldehyde for 20 min, washed in PBS three times (5 min each), and permeabilized with 0.5% Triton X-100 in PBS for 10 min. After quenching with PBS-glycine three times (10 min each), samples were blocked in 1% BSA/0.1% saponin in PBS for 1 h, then incubated with antibodies in block overnight at 4°C. After washing three times with block buffer (10 min each), secondary antibodies were added for 1 h at ambient temperature. The samples were washed with block buffer once for 15 min and with PBS three times (10 min each), then were mounted with Prolong Gold antifade reagent with DAPI (Invitrogen). The slides were allowed to dry overnight and then sealed with nail polish.

Measurement of intracellular Ca^{2+}

MDCK cells cultured on glass bottom dishes (MatTek Corporation) were transfected with control or OCRL1 siRNA. Ca^{2+} measurements were performed 2 days after transfection.

Cells were loaded with Fura-2AM (5 μ M) (Invitrogen) in HEPES-buffered saline (HBS; 135 mM NaCl, 4.5 mM KCl, 2.5 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, 10 mM glucose, pH 7.4) for 2 h at room temperature. Fura-2AM loaded cells were washed with HBS and incubated for an additional 10 min to allow for further de-esterification of the dye. Glass bottom dishes were mounted in a QE-1 imaging chamber (Warner Instruments). The temperature of the chamber was maintained at 37°C with a dual channel bipolar temperature controller (model TC-344B, Warner Instruments). Ca²⁺ imaging was performed using an inverted Nikon Eclipse Ti microscope equipped with an ORCA-Flash 2.8 camera (Hamamatsu). For excitation we used a PhotoFluor II metal halide light source (89 North) connected to a Lambda 10–3 filter wheel system (Sutter Instruments) with 340 nm and 380 nm filters (Chroma Technology). Images were acquired and analyzed with NIS-elements software (Nikon). *In situ* calibration with ionomycin (5 μ M) was performed to estimate Ca²⁺ calibration parameters under zero or saturating intracellular Ca²⁺ concentrations. The zero-free Ca²⁺ solution contained 135 mM NaCl, 4.5 mM KCl, 2 mM EGTA, 1 mM MgCl₂, 10 mM HEPES and 10 mM glucose, pH 7.4. HBS was used to estimate Fura-2 ratio values at saturating Ca²⁺ concentrations. Ten cells were randomly selected for analysis per experiment.

Supplementary Material

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Acknowledgments

We thank Robert Nussbaum and Sharon Suchy for their generous gift of anti-OCRL1 antibody and Melanie Warnes for experimental support. This work was supported by grants from the NIH (DK064613) and the Lowe syndrome Association to O. A. W. H. F. was supported by NIH grant GM070736. We are grateful to the Urinary Tract Epithelial Imaging, Model Organisms, and Single Tubule Physiology Cores of the P30 Pittsburgh Center for Kidney Research (NIH DK079307) for technical support.

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Figure 1. OCRL1 is expressed in the zebrafish kidney, eye and brain

A) RT-PCR was performed using *ocr1*-specific primers to detect expression of *ocr1* in embryo extracts collected at the 13-somite stage (13ss), 24 hpf and 48 hpf. B) *In situ* hybridization for *ocr1* expression was performed on embryos collected at the 6-somite stage (6ss), 13ss, 24 hpf and 48 hpf. No expression was observed at the 6ss. At the other stages, expression of *ocr1* in the brain (B), eye (E) and kidney (K) is marked by arrowheads. C) Higher magnification images of the pronephric kidney distal tubule and duct are shown to highlight *ocr1* expression in this region. D) Representative images of embryos at 24 hpf and 48 hpf incubated with *ocr1* sense probe are shown as a negative control.

Figure 2. *Ocr1* morphants develop pericardial edema and body curvature

A) Following MO injection, 48 hpf zebrafish larvae were classified into three categories: class I, wild type; class II, moderate body curvature and mild pericardial edema; and class III, severe body curvature and severe pericardial edema. Representative images are shown.

B) Embryos were injected with the indicated dose of control or *ocr1* MO or left uninjected and classified as above at 48 hpf. At 7.5 ng, all the embryos were dead at 24 hpf. For rescue studies, embryos were injected with mRNA encoding wild type *ocr1* or the phosphatase-dead R559G mutant, followed by injection with 6.0 ng of *ocr1* MO. Phenotypes were classified at 48 hpf. The graph shows quantitation of three independent injections with at least 50 injected embryos per dose in each experiment, except for R559G, which represents data from 64 embryos.

Figure 3. Kidney function is disrupted after *ocr1* knockdown

A) 1 ng of 10 kDa Rhodamine dextran was injected into the common cardinal vein of control embryos ($n = 32$) or moderately (class II) affected *ocr1* morphants ($n = 25$; all injected with 6 ng MO) at 24 hpf. Images were acquired at 1, 5 and 24 h post-injection. B) The progressive loss of fluorescence near the cardinal vein area (white box at 0 h) was quantified and plotted as described in Materials and Methods.

Figure 4. Cilia are elongated in the proximal tubule of *ocr1* morphants

Zebrafish embryos injected with 6 ng control or *ocr1* MO were fixed and processed at 24 hpf for indirect immunofluorescence to detect acetylated α -tubulin (green) and IFT88 (red). Representative images of pronephric kidney proximal tubules in control and *ocr1* morphant larvae are shown and cilia length is plotted below (mean $\pm$ SEM from four embryos per condition; >100 cilia quantitated per condition). * $p < 0.001$ by Student's *t* test. Scale bar: 5 μ m.

Figure 5. Knockdown of OCRL1 in renal epithelial cells causes elongation of cilia

A) Filter-grown MDCK cells treated with siRNA targeting OCRL1 or control siRNA were solubilized and blotted to detect OCRL1 and β -actin (as a loading control). We typically observed 50–95% knockdown of OCRL1 in our experiments. B) Filter-grown MDCK cells treated with control or OCRL1 siRNA were fixed and processed for indirect immunofluorescence to detect acetylated tubulin. Cells were viewed by confocal microscopy to visualize cilia. Bar: 10 μ m. C) Individual cilia lengths (at least 100 per sample) were measured from random fields of cells treated with control or OCRL1 siRNA, sorted in ascending order by length and plotted from shortest to longest. Each circle in the graph represents the length of a single cilium. The median cilia length is 3.38 μ m versus 1.72 μ m for cells treated with OCRL1 versus control siRNA, respectively, and is statistically different in the two samples (rank sum test $p < 0.001$). D) Scanning electron microscopy of MDCK cells treated with control or OCRL1 siRNA shows no obvious difference in cilia morphology. Two representative images for each condition are shown. Bar: 1 μ m.

Figure 6. OCRL1 knockdown has no effect on cilia length in fibroblasts

A) Western blot of OCRL1 and β -actin from human fibroblasts treated with control or OCRL1 siRNA. B) Cilia length was measured in human fibroblasts treated with control or OCRL1 siRNA after 72 h serum starvation and plotted as described in the legend to Figure 5. Median cilia length was identical (4.8 μ m) in control and OCRL1-depleted cells. C) Cilia length was measured after 4 h of serum starvation. Median cilia length was 4.7 μ m in control and 5.3 μ m in OCRL-depleted cells.

Figure 7. OCRL1 knockdown disrupts cyst morphogenesis

A) MDCK cells were transfected with control or OCRL1 siRNA and plated in Matrigel. Cells were fixed and processed for indirect immunofluorescence after 6 days to visualize nuclei (blue), acetylated tubulin (green) and ZO-1 (red). B) Cilia length was quantitated in MDCK cysts treated with control, OCRL1 or galectin-3 siRNA. C) Cyst morphology was quantitated in cells treated as in (B). Cysts with multiple or collapsed lumens were considered to be aberrant. Representative examples of a normal and abnormal cyst stained with anti-acetylated tubulin are shown. The average $\pm$ SD of 4–5 experiments for each condition is plotted (100 cysts counted per condition per experiment). * $p < 0.001$ versus control siRNA by Student *t*-test. Scale bars: 10 μm .

Figure 8. OCRL1 depletion reduces ATP-stimulated calcium mobilization

Confluent MDCK cells cultured on glass bottom dishes were transfected with control or OCRL1 siRNA. Ca^{2+} imaging was performed as described in Materials and Methods. A) ATP-stimulated calcium mobilization in control and OCRL1-depleted MDCK cells. Time course of change in $[Ca^{2+}]_i$ in control and OCRL1 depleted cells. The time of addition of ATP (100 μM) is indicated with an arrow. Data represent the average from 9 to 10 independent experiments. B) Peak intracellular calcium following ATP addition in control and OCRL1 siRNA-treated cells. Data represent the mean $\pm$ SEM from 9 to 10 independent experiments. *p = 0.023 by Mann-Whitney.