The Lowe syndrome protein OCRL1 is involved in primary cilia assembly

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Lowe syndrome (LS) is a devastating, X-linked genetic disease characterized by the presence of congenital cataracts, profound learning disabilities and renal dysfunction. Unfortunately, children affected with LS often die early of health complications including renal failure. Although this syndrome was first described in the early 1950s and the affected gene, OCRL1, was identified more than 17 years ago, the mechanism by which Ocrl1 defects lead to LS’s symptoms remains unknown. Here we show that LS display characteristics of a ciliopathy. Specifically, we found that patients’ cells have defects in the assembly of primary cilia and this phenotype was reproduced in cell lines by knock-down of Ocrl1. Importantly, this defect could be rescued by re-introduction of WT Ocrl1 in both patient and Ocrl1 knock-down cells. In addition, a zebrafish animal model of LS exhibited cilia defects and multiple morphological and anatomical abnormalities typically seen in ciliopathies. Mechanistically, we show that Ocrl1 is involved in protein trafficking to the primary cilia in an Rab8- and IPIP27/Ses-dependent manner. Taking into consideration the relevance of the signaling pathways hosted by the primary cilium, our results suggest hitherto unrecognized mechanisms by which Ocrl1 deficiency may contribute to the phenotypic characteristics of LS. This conceptual change in our understanding of the disease etiology may provide an alternative avenue for the development of therapies.

INTRODUCTION

Cereborenal (CR) developmental diseases are characterized by functional abnormalities in the brain and kidneys (1). Although these pathologies display a broad range of specific symptoms and severity, they also share multiple phenotype characteristics (1). Furthermore, in recent times, it has become increasingly evident that many genetic diseases with similar phenotypic manifestations are linked by functional abnormalities in common organelles or pathways (2).

Important examples of CR diseases include Lowe syndrome (LS) and a group of pathologies collectively known as ciliopathies (1). On the one hand, LS is a developmental disease characterized by abnormal function of the Inositol 5-phosphatase (EC 3.1.3.36) Ocrl1 [Oculo-Cerebro-Renal syndrome of Lowe protein 1 (3)]. The mechanisms by which deficiencies in Ocrl1 lead to the CR manifestations of this disease, however, still remain elusive. On the other hand, ciliopathies are a genetically heterogeneous group of pathologies [including Joubert and Bardet–Biedl syndromes (BBSs)] in which the function of the primary cilium is compromised (4). The primary cilium is a non-motile, axoneme-based organelle present in most cells that host important sensory machinery (5). In consequence, cells from ciliopathy patients display defects in a variety of signaling pathways critical for processes such as cell migration, differentiation and cell cycle regulation (5). Importantly, primary cilia abnormalities have been linked to the brain, eye and kidney abnormalities (e.g. Joubert and Meckel syndromes) similar to LS patients (4).
In fact, the parallels between LS and ciliopathies extend further: specifically, we recently demonstrated that LS patient’s cells are deficient for cell migration/spreading (6). Interestingly, cell migration/spreading defects have also been observed in other CR diseases (1), particularly in BBS (7). In addition, Ocr1 is known to interact with the GTPase Rab8 (8) which in turn has a crucial role in ciliogenesis (9). Interestingly, one variant of BBS involves defects in the function of Inpp5E, another Inositol 5-phosphatase with substrate specificity similar to Ocr1 (10,11). Finally, it should be noted that Ocr1 contains an ASPM, SPD-2, Hydin (ASH) domain (12) which is present in ciliary proteins (13).

A putative conceptual connection between LS and ciliopathies is an attractive possibility because it would indicate that primary cilia dysfunction contributes to the elusive mechanism behind LS. However, and despite the similarities between these pathologies, no formal link has been found between LS and ciliopathies.

The present work fills this gap by demonstrating that cells from LS patients share some characteristics observed in cells from ciliopathy patients. Specifically, we found that cells from LS patients display defects in the assembly of the primary cilium. These abnormalities were reproduced in cell lines using siRNA against Ocr1. Importantly, a zebrafish model for Ocr1 deficiency displayed embryonic phenotypes characteristic of ciliopathies. We also established that mechanistically, Ocr1 participates in protein trafficking towards the primary cilium in a Rab8/IPIP27-dependent manner.

This novel viewing links LS with other CR syndromes could provide a breakthrough in terms of mechanistic insights or the design of novel therapeutic approaches against LS.

RESULTS

LS and primary cilia abnormalities

LS patient’s cells display defects in primary cilium assembly. Given the similarities between LS and ciliopathies, we tested whether LS patients’ cells display normal primary cilium. Therefore, we induced the formation of primary cilium, by serum starvation in fibroblasts taken from normal individuals and from two unrelated LS patients (see Materials and Methods and 6). The presence of primary cilium was assessed by immunofluorescence using anti-acetylated tubulin and anti-pericentrin antibodies (Fig. 1A). Cells from both LS patients showed defects in primary cilium formation as shown by the lower proportion of cells displaying primary cilium and by a marked decrease in primary cilium length when compared with their normal counterparts (Fig. 1A and B). Indeed, our results indicate that, after 12 h starvation, compared with the control <50% of LS cells displayed primary cilium which on average were half the length of those observed in normal fibroblasts (Fig. 1B). Our data also showed that upon serum addition the primary cilium collapsed similarly in both LS and normal cells (Fig. 1C). Importantly, the primary cilium abnormalities were rescued by transfection with green fluorescent protein (GFP)–Ocr1 (Fig. 2A and B). Taken together, these results suggest that LS patients’ cells are less efficient for assembly of primary cilia.

In support of our findings, knock-down of Ocr1 using specific siRNAs in NIH3T3 cells reproduced the primary cilia assembly defects observed in patients’ cells (Fig. 2C and D). Importantly, transfections with siRNA resistant, human GFP–Ocr1 rescued the primary cilium abnormalities, ruling out knock-down off-target effects (Fig. 2D).

Zebrafish ocr1 morphants display developmental abnormalities typically associated with primary cilia assembly defects. Attempts to create a mouse model of LS highlighted fundamental differences in the compensatory mechanism to cope with Ocr1 deficiencies between humans and mice (14,15). Since these differences have constituted an obstacle for the establishment of mice models for LS, we utilized zebrafish. Moreover, this organism has been successfully used to reproduce the anatomical and morphological anomalies associated with ciliopathies (16,17). To establish whether ocr1-depleted zebrafish presented ciliopathy-like defects we ‘knocked-down’ ocr1 expression using an atg and a splice antisense morpholino (MO). The splice MO targeted the splice site between exons 1 and 2, adding intron 1 to the resulting transcript (Supplementary Material, Fig. S1A and B). The suppression of ocr1 expression was confirmed by western blot (Supplementary Material, Fig. S1C and D) and its specificity corroborated by our ability to rescue the morphant phenotype by injecting ocr1 mRNA (Supplementary Material, Fig. S1E–G).

Forty-eight hour post fertilization (h.p.f.), ocr1 morphants exhibited smaller eyes, short and curved bodies, small head and fewer, mislocalized melanophores (Fig. 3A and B). Furthermore, 6 days post fertilization (d.p.f.), morphants displayed anterior pronephros dilatation and cystogenesis, features observed in other ciliopathy morphants/mutants (Fig. 3C). At this stage, the anterior part of the skull was flattened as the head structures were not formed properly. The viability of the morphants was lower than the controls and only 26% survived 6 d.p.f. (Fig. 3D). Hematoxylin and eosin (H&E) stained sections of the head showed reduced levels of cartilage, neural tissue (Supplementary Material, Fig. S1H) and disorganization of the retinal layers in both eyes.

The zebrafish pronephros are lined with motile cilium that facilitate excretion, whereby the development of a cyst usually indicates defective fluid flow. Upon labeling the cilia for acetylated tubulin (axoneme) and gamma tubulin (basal bodies) in ocr1 morphants, we observed gross disorganization and shorter primary cilium compared with the un.injected control embryos (Fig. 4A). Laterality defects are often observed in ciliopathies. We quantified heart looping by in situ hybridization using a cardiac marker, cmlc2 (cardiac myosin light chain 2) (18). Forty-three percent of ocr1 morphant embryos exhibited laterality defects with 19.6% of embryos displaying right-sided heart looping and 13.6% with medial or ambivalent heart position instead of the expected leftward looping found in all controls (Fig. 4D).

Some ciliopathy patients display facial dysmorphology as a result of likely aberrant neural crest cell (NCC) migration (16). Upon staining with Alcian blue to delineate the cranial cartilage structures, ocr1 morphants had a much lower intensity of blue staining compared with controls at the same stage (Fig. 4C). Both the palatoquadrate and Meckel’s cartilage, components of the mandibular arch, were short and malformed in the morphants.
and the pharyngeal arches were reduced in size or absent. Furthermore, neural crest-derived melanophores were fewer and mislocalized (Fig. 3B), further supporting the NCC origin of the phenotype. To further assess that the cranial cartilage structures and melanocyte migration defects are due to NCC misguidance, we injected Sox10–GFP zebrafish embryos with ocrl1 MO. We observed a clear mispatterning in NCC migration, where the streams of cells were migrating randomly (Supplementary Material, Fig. S1I). Defects in ciliogenesis are also observed in an ocrl1 germline mutant strain (Barinaga-Rementeria Ramirez et al., submitted for publication), in the pronephric duct and neuromasts (Fig. 1K). This confirms the specificity of the ciliogenesis defect and indicates its prevalence in other ciliated tissues in addition to the pronephric duct.

In summary, zebrafishocrl1morphants display characteristics commonly found in ciliopathies (including shortened cilia, laterality and NCC patterning defects) and also in LS (e.g. facial dysmorphism and cell migration defects).

**Role of Ocrl1 in primary cilia assembly**

Since Ocrl1 deficiencies led to ciliopathy-like phenotypes, we next questioned how this protein participates in ciliogenesis. Since evidence points to a role of Ocrl1 in vesicle trafficking (3,12,19,20), we tested whether this protein is involved in cargo sorting into the primary cilium.

Ocrl1-deficient cells show vesicle trafficking deficiencies to the primary cilium. To address whether knock-down of Ocrl1 affected protein trafficking to the primary cilium, we depleted Ocrl1 using specific siRNAs and analyzed the fluorescence intensity ratio between cargoes (i.e. Rhodopsin or Inversin) and

![Figure 1.](https://example.com/figure1.jpg)
acetylated tubulin within the primary cilium in cells that still bear cilia (10–20% cells). We observed a significant decrease in the amount of Rhodopsin-red fluorescent protein (RFP) localized to the primary cilium in Ocrl1-depleted cells when compared with the control (Fig. 5A). Further, trafficking to the primary cilium of a CD25 chimera bearing Rhodopsin’s primary cilium-sorting signal was also affected by Ocrl1-depletion (see below). In contrast, Inversin–GFP, a cytosolic protein that does not require vesicle trafficking for primary cilium localization, showed no difference in its ability to reach the primary cilia in Ocrl1 knock-down and control cells (Fig. 5B). These results strongly suggest that Ocrl1 participates in vesicle trafficking to the primary cilium.

Ocrl1 partially co-localizes with pericentrin at the base of the primary cilium and with endosomal markers. Ocrl1 function in intracellular traffic is likely linked to its intracellular localization during primary cilia assembly. In general, Ocrl1 displayed its typical perinuclear localization but it was also found as puncta and 25% of the cells displayed a well-defined Ocrl1-positive structure at the primary cilium base. This Ocrl1 primary cilium-basal compartment co-localized with the endosomal/basal body marker pericentrin (PCNT, Fig. 6A). Interestingly, this primary cilium-basal Ocrl1 structure also strongly co-localized with the Ocrl1-interacting protein IPIP27/Ses (Fig. 6B). The IPIP27A and Appl1 endosomal proteins also showed overlap with Ocrl1 in more peripheral puncta (Fig. 6B, C). It should be noted that although we use the term ‘F&H proteins’ [due to the presence of an FxxxH Ocrl1-binding motif (21,22)] to refer to both IPIP27 and Appl1, these proteins have different functions and bind Ocrl1 in a mutually exclusive manner (21,22).

In addition, Ocrl1 partially co-localized with the Rab8 GEF, Rabin8 within peripheral structures (Fig. 6D). It should be highlighted that Rabin8 and its target, the Ocrl1-interacting protein Rab8, have been shown to play an important role in
primary cilia assembly (9,23). Indeed, we found that upon overexpression of Rab8, Ocrl1 was detected within the primary cilium (Supplementary Material, Fig. S2A). It should be highlighted that Ocrl1 normally did not localize to the primary cilium. Furthermore, co-overexpression of Ocrl1 and Rab8 led to the formation of a membrane bulge in the primary cilium (Supplementary Material, Fig. S2B), typically observed upon abnormal vesicle trafficking to this organelle (24). Importantly, the Ocrl1G664D Rab-binding mutant was unable to trigger this primary cilium assembly anomaly (Supplementary Material, Fig. S2C). Although the physiological relevance of these observations is unclear (we have not observed ciliary localization of Ocrl1 under other conditions), they indicate a link between Ocrl1 and the Rab8 primary cilium assembly pathway. In our hands, with the exception of Rab8 and 5 (see below), other Rabs tested showed negligible co-localization with Ocrl1 under primary cilium assembly conditions (data not shown).

Ocrl1 co-localizes with internalized TAC-VxP primary cilia-reporter. Since IP27s and Appl1 are endocytic proteins, we tested whether the endocytic pathway also contributes to protein trafficking to the primary cilium in an Ocrl1-dependent manner.

Chimeras between the extracellular and transmembrane domains of TAC (interleukin-2 receptor α-subunit or CD25) and different sorting signals have been successfully used to study vesicle-trafficking pathways (25,26). Due to the availability of highly specific anti-TAC extracellular domain antibodies [e.g. 7G7 (25)], these constructs have been invaluable for the analysis of the endocytic pathway (25,26). We designed a TAC chimeric primary cilium sorting-reporter protein bearing the VxP primary cilium-targeting signal of human rhodopsin [QVSPA (27)] and verified that this TAC–VxP chimera accumulates in the primary cilium (Supplementary Material, Fig. S3). As expected, similar to Rhodopsin (Fig. 5), TAC–VxP also showed deficient localization to the primary cilium upon Ocrl1 knock-down (Supplementary Material, Fig. S3). Next, we investigated whether the internalized TAC–VxP fraction co-localized with Ocrl1 and with a third protein, e.g. IP27. As shown in Fig. 7, under primary cilia-induction conditions, the internalized TAC–VxP chimera co-localized with Ocrl1 within intracellular structures. Importantly, ~25% of these puncta also co-localized with the endosomal proteins IP27 or Appl1 (Fig. 7A and B). Further, Ocrl1 localization in TAC-containing early endosomes under primary cilia-induction conditions was confirmed by triple-co-localization with Rab5 (Fig. 7C). In contrast, Rab8 did not co-localize with the internalized chimera (Fig. 7D).

Taken together, these results indicate that Ocrl1 also participates in primary cilium-targeting of proteins via the endocytic pathway.

Ocrl1 function in ciliogenesis relies on ASH–RhoGAP domain-mediated interactions. Since co-localization and TAC–VxP trafficking experiments (Figs 6 and 7) suggested a link between Rab8/F&H proteins and Ocrl1 during ciliogenesis, we tested whether the interaction of Ocrl1 with these proteins was required for primary cilium assembly. Specifically, we assessed the ability of different siRNA-resistant Ocrl1 mutants to support ciliogenesis in Ocrl1-depleted cells. Our results showed that mutations that impair interaction with either Rab8 or F&H proteins render Ocrl1 unable to sustain primary cilia assembly (Fig. 8A).

We also reasoned that, since Ocrl1 depletion by siRNA-mediated knock-down typically leaves 10–20% residual Ocrl1, increasing the intracellular dose of its interaction partners (i.e. IP27A/Rab8) will raise the levels of Ocrl1-bound complexes by mass action. We predicted that, if these Ocrl1 complexes play a role in ciliogenesis, then the overexpression of IP27A or Rab8 should lead to phenotype alleviation [by a ‘high-copy suppression’-like mechanism (28)]. Indeed, overexpression of either IP27A or Rab8 ameliorated the primary cilium phenotype due to Ocrl1 depletion (Fig. 8A). Furthermore, complementary experiments showed that Ocrl1 mutants unable to bind Rab8 (G664D; 8,29) as well as an IP27A construct lacking its PH domain (mislocalized to the cytosol—data not shown), exerted a dominant-negative effect inhibiting ciliogenesis (Fig. 8B). Moreover, as described...
above, Rab8 binding by Ocrl1 was required to induce the ‘bulged’ primary cilia phenotype (Supplementary Material, Fig. S2C). As a whole, these results suggest that Ocrl1 is functionally linked to Rab8/IPIP27 for primary cilia assembly. In contrast, a previously described clathrin- and AP2-binding mutant (30) rescued ciliogenesis in knock-down cells (Fig. 8A). These results suggest that Ocrl1 function in primary cilia assembly involves a role in endosomal trafficking rather than in plasma membrane uptake. Further, the close Ocrl1 homolog, Inpp5b [capable of binding Rab8/F&H proteins but not clathrin or AP2 (30)], partially rescued the primary cilia phenotype displayed by Ocrl1 knock-down cells (Fig. 8A).

Importantly, key results described in this work were also reproduced in the human retinal cell line RPE1 (Supplementary Material, Fig. S4).

**DISCUSSION**

The contribution of this study to the field of CR diseases is two-fold: (i) it demonstrates that the Ocr syndrome of Lowe is not an isolated disease but it shares cellular phenotypes with the heterogeneous category of ciliopathies and (ii) it reveals a novel cellular role for Ocrl1 in cilia assembly. Specifically, we showed that Ocrl1 participates in ciliogenesis by contributing to protein trafficking to this organelle in an Rab8/IPIP27-dependent manner (Fig. 8C).

**LS shares cellular phenotypes with ciliopathies**

This novel view of LS is supported by the following evidence:
Primary cilia assembly abnormalities occur in LS patient cells (and can be reproduced in cell lines by Ocrl1-depletion—Figs 1 and 2).

A zebrafish model of Ocrl1 deficiency displays some phenotypes also seen in ciliopathy models (Figs 3 and 4).

Ocrl1 has a ciliary function dependent on its ASH-domain interaction with the ciliogenesis protein Rab8 (Fig. 8A and Supplementary Material, Fig. S2).

Spreading and migration phenotypes are observed in both ciliopathy and LS patient cells (1,6).

Common organs are affected in ciliopathies and LS. Nevertheless, LS and ciliopathies differ in the specific tissue involvement within the same organ (e.g. cataracts versus retinitis pigmentosa) which remains to be explained. Likewise, LS patients do not develop renal cysts but have renal tubulopathy as seen in several ciliopathies (e.g. BBS and Nephronophthisis). It is conceivable that abnormalities at different points within a pathway/organelle would lead to different physiological deficiencies. Furthermore, substantial genetic heterogeneity has been observed in that mutations affecting a single gene can lead to different phenotypic characteristics. For example, mutations in CEP290 can give rise to Nephronophthisis, Bardet–Biedl, Meckel or Joubert syndromes, each a clinically distinct with key overlapping features (31). Similarly, OCRL1 mutations can cause both LS and Dent-2 disease (32). In addition, ciliogenesis defects may account for only part of the LS pathology along with other vesicle trafficking-related defects [e.g. clathrin-binding dependent, see (6, Barinaga-Rementeria Ramirez et al., submitted for publication)].

Role of Ocrl1 in primary cilia assembly

At the cellular level, our data suggest that Ocrl1 participates in protein trafficking to the primary cilium in an F&H protein/Rab8-dependent manner (Fig. 8C). Specifically, efficient targeting of Rhodopsin–RFP and a primary cilia-localized TAC-chimera was Ocrl1 dependent. Moreover, the ability of Ocrl1 to rescue primary cilia assembly depended on its interactions with Rab8 and with the F&H endocytic proteins and partially relied on endocytic routes for primary cilia protein localization.

It has been established that Appl1 and IPIP27 form exclusive and sequential sub-complexes with Ocrl1 (with likely different functions), but little is known about the interactions and physiological function of F&H proteins (8,21,22,33). Thus, although Appl1 participates in several signaling pathways (34,35), no direct involvement in ciliogenesis for this protein has been reported before. However, it has been shown that the Appl1-interaction partner Rab5 participates in vesicle trafficking to the primary cilium (36). Interestingly, co-localization between Ocrl1, Rab5 and TAC-VxP was observed following chimera internalization (Fig. 7C). In addition, the IPIP27/Ses proteins have been recently shown to play a role in membrane recycling pathways (21), which in turn are known to be involved in primary cilium assembly (9). Moreover, IPIP27A strongly co-localized with Ocrl1 at the primary cilium base, its overexpression rescued the primary cilium defect due to Ocrl1 depletion and the dominant-negative construct IPIP27AΔPH interfered with primary cilium assembly. Nevertheless, details of the function of F&H proteins in primary cilium assembly will need to be established before we can further our understanding of the relative relevance of Appl1 and IPIP27 in ciliogenesis. Although a study centered on the interactions of F&H proteins and their role in primary cilium formation lies beyond the scope of this report, it constitutes the focus of ongoing research in our labs.

In contrast, the role of Rab8 in primary cilium assembly is better characterized (23). Indeed, a defect in ciliogenesis, very similar to the one displayed by LS patients’ cells, is observed upon defective Rab8 activation due to abnormal ciliary membrane localization by BBS proteins (23).
Interestingly, Ocrl1 membrane association requires interaction with Rab GTPases and one of the direct interactors of Ocrl1 is Rab8 (8,37). In fact, co-overexpression of Rab8 and Ocrl1 promoted the formation of a membrane bulge within the primary cilium. Interestingly, this effect on the primary cilia morphology was dependent on the Ocrl1–Rab8 interaction.

Importantly, in agreement with Inpp5b ability to interact with both F&H proteins and Rab8, overexpression of this Ocrl1 homolog led to partial rescue of the primary cilia assembly defects due to Ocrl1 depletion (Fig. 8A). These results are consistent with the observation that Ocrl1 binding to clathrin and AP2 (absent in Inpp5b) was not required for rescuing ciliogenesis (Fig. 8A). However, it should be highlighted that LS patient fibroblasts used in this study display normal levels of Inpp5b (6) in spite of primary cilia assembly defects (Fig. 1). This observation suggests that from these two homologous phosphatases, Ocrl1 is the main contributor to ciliogenesis. Nevertheless, our work is compatible with the notion that there is partial functional redundancy between Inpp5b and Ocrl1 for primary cilia assembly.

Our working model (Fig. 8C) proposes that proteins targeted to the primary cilia, follow two routes. On the one hand, a direct pathway involving Ocrl1 and its interaction partner Rab8 targets cargo-containing carriers from the TGN/recycling endosome to the primary cilium. On the other hand, an indirect route delivers internalized cargo to the primary cilium by an Ocrl1- and F&H-dependent mechanism. Taking into account what is known about F&H proteins (21,22), we speculate that internalized primary cilia-targeted cargo will first pass through an Appl1-containing compartment, to then move into an IPIP27-positive recycling endosome (21) from where it will be targeted to the primary cilium. Interestingly, Rab8 is also found in recycling endosomes (9,38). Thus, although we have not observed triple co-localization of the internalized TAC–VxP reporter and Ocrl1 with Rabin8/Rab8, we cannot rule out that both routes...
converge into a common recycling compartment to deliver cargo to the primary cilium.

**Perspectives**

The results reported in this study support a novel view of LS involving primary cilia assembly defects. These findings may yet provide new avenues for the further investigation of this disease pathomechanisms. In addition, we speculate that in the future this concept will influence the design of therapeutic strategies against LS, perhaps leading to the adoption or adaptation of approaches currently available for ciliopathy treatment.

**MATERIALS AND METHODS**

**Reagents**

DAPI was obtained from Molecular probes. Other materials were purchased from Fisher Scientific (Fairlawn, NJ,
USA) or Sigma (St. Louis, MO, USA) unless noted otherwise.

Plasmids and antibodies
DNA constructs were prepared using standard techniques, site-directed mutagenesis was performed using a QuikChange kit according to manufacturer’s protocols (Stratagene, La Jolla, CA, USA). DNA plasmids and antibodies used in this study are described in Supplementary Material, Tables S1 and 2, respectively.

Cells and cell culture
Normal, LS1 and LS2 primary dermal fibroblasts (GM07492, GM 01676 and GM 03265, respectively) were obtained from the NIHGMS Human Genetic Cell Repository (Coriell Institute for Medical Research, Camden, NJ, USA). LS1 patient cells bear an OCRL1R827X mutation that renders an unstable Ocrl1-mutated protein that cannot be detected by western blotting with a specific antibody. Therefore, we considered LS1 to be effectively Ocrl1 null. In contrast, LS2 cells show dramatic expression defects of a WT protein, barely detectable by western blot.

RPE-1 and NIH3T3 cell lines were acquired from ATCC. Human Dermal Fibroblasts and NIH3T3 were cultured as described (6). RPE-1 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM), streptomycin/penicillin, 2 mM L-glutamine, 10% fetal bovine serum (FBS) and 0.01 mg/ml hygromycin B (BD Biosciences).

Cell transfections
siRNA/plasmid co-transfections. RPE-1 or NIH3T3 were seeded in 4 cm² cell culture plates and grown to 70% density in 24 h. Media was then exchanged for DMEM without serum and antibiotics 1 h before transfection. Cells were co-transfected with TransIT-LT1 (Mirus) complexed with 0.5 μg of plasmid and TransIT-TKO (Mirus) complexed with 40 pmol of siRNA according to the manufacturer’s protocol. siRNAs include anti-Ocrl1 (Santa Cruz: sc-39074). The procedure was repeated twice, at 96 and 72 h before use. Complete media was added 12 h after each transfection.

Plasmid transfections. RPE-1 and NIH3T3 cells were transfected by using TransIT-LT1 (Mirus) according to the manufacturer’s protocol. Transfection of primary fibroblasts was achieved by electroporation as described before (6). Cells were normally used 24–48 h after transfection.

Protein expression levels were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting was performed as described previously (6).
Cell staining and microscopy
In all cases, stained cells were imaged in a Zeiss Axiovert-200M microscope equipped with temperature-controlled, motorized stage for optical z-sectioning.

Immunofluorescence. Cells fixed in 4% formaldehyde/ phosphate buffer saline (PBS) were processed for immunofluorescence by incubating with primary antibodies in complete media containing 0.1% saponin (unless indicated otherwise) for 60 min at room temperature. After washing with PBS, cells were incubated with secondary antibodies in media supplemented with saponin for 45 min. Coverslips were washed with PBS and mounted on slides using Aqua-PolyMount (Polysciences) and imaged.

DAPI staining. DAPI stain was diluted to 1:60 000 in PBS and cells were stained for 20 min at room temperature.

Ciliogenesis assays
Cells were seeded onto 4 cm² glass coverslips and grown for 12–24 h in complete media. Then media was exchanged with 0–0.1% serum DMEM (starvation media) to induce ciliogenesis for indicated times. Human dermal fibroblasts and RPE-1 cells were typically starved for 6–8 h, whereas NIH3T3 cells were typically starved for 3–4 h. Cells were then fixed for 10 min in 4% formaldehyde–PBS and immunolabeled with anti-acetylated tubulin and/or indicated specific antibodies. Ciliogenesis was quantified using fluorescence microscopy under ×40 objective by scoring the percentage of total cells containing primary cilia marked by acetylated tubulin. Since we observed some variability in-between experiments, we adopted a ‘relative to normal’ representation of the results. The error of each determination was propagated according to standard (39):

\[
\Delta \left( \frac{LS}{N} \right) = \left( \frac{\Delta LS}{LS} \right) + \left( \frac{\Delta N}{N} \right)
\]

where the relative error of the ratio equals the sum of the relative errors of denominator and numerator.

The cilia length was determined by imaging acetylated tubulin-immunolabeled primary cilia under a ×100 objective and tracing the length of the cilia tip-to-tip using ImageJ’s segmented line tool.

Quantification of protein trafficking to the cilium
Following co-transfection plasmid DNA and siRNAs, NIH3T3 cells were seeded on glass coverslips and ciliogenesis induced as described above. After fixation, when required, cells were immunolabeled with 7G7 anti-TAC monoclonal antibody in the absence of saponin for detection and quantification of the primary cilia. Cells were then immunolabeled with anti-acetylated tubulin antibody in the presence of saponin that was used as a reference marker. Image J-z-stacks were collected under a ×100 objective and z-projections were made using ImageJ. The size, mean intensity and mean background intensity in the channels corresponding to both cargo and acetylated tubulin were measured within the cilia. Total fluorescence intensity was calculated by multiplying the region of interest area by the difference between ciliary and background mean signal intensity. The ratio between the fluorescence signals of cargo to acetylated tubulin was calculated.

TAC–VxP internalization experiments
NIH3T3 cells were transfected with the indicated plasmids and then seeded onto glass coverslips. Ciliogenesis was initiated by starvation for 2 h. Coverslips were then placed in 10°C PBS and immunolabeled for surface TAC–VxP using 1:30 7G7 antibody in DMEM at 10°C for 45 min. Coverslips were rinsed with ice-cold PBS and then incubated in 37°C starvation media for 20 min to allow TAC–VxP internalization. After the internalization period, residual antibody was removed by acid wash when indicated as described previously (6), fixed and immunolabeled with secondary antibody in the presence of saponin.

Zebrafish handling and experiments
Wild-type (AB × Tup LF) zebrafish and Tg (Sox10:gfpl) were maintained and staged as described previously (40). Generation of the ocrl1 germine mutant is described elsewhere (Barinaga-Rementeria Ramirez et al., submitted for publication). Antisense MO oligonucleotides (Gene Tools) were designed against the start codons of ocrl1 and against the exon 1–intron 1 splice site of ocrl1. MO sequences are CGGAAATTCCTAATGAGGTTCAT for the translation blocking target (atg) and AGCCTCAAATAAGGA-TACGGCTA from the splice junction target.

MOs were injected (2–3 ng) into embryos at the 1- to 2-cell stage and incubated at 28.5°C until the desired stage. The specificity of splice MOs was confirmed by RT–PCR. RNA was extracted from 25 morphants and 25 controls at 48 h.p.f. using the TRIzol (Invitrogen) method of RNA extraction and precipitation. First-strand cDNA was synthesized using random primers (Sigma-Aldrich) and Omniscript transcriptase (QIAGEN), according to the manufacturer’s instructions. Standard PCR was carried out using primers to sequences specific for splice MOs was confirmed by RT–PCR. RNA was extracted from 25 morphants and 25 controls at 48 h.p.f. using the TRIzol (Invitrogen) method of RNA extraction and precipitation. First-strand cDNA was synthesized using random primers (Sigma-Aldrich) and Omniscript transcriptase (QIAGEN), according to the manufacturer’s instructions. Standard PCR was carried out using primers to sequences specific for splice MOs was confirmed by RT–PCR. RNA was extracted from 25 morphants and 25 controls at 48 h.p.f. using the TRIzol (Invitrogen) method of RNA extraction and precipitation. First-strand cDNA was synthesized using random primers (Sigma-Aldrich) and Omniscript transcriptase (QIAGEN), according to the manufacturer’s instructions. Standard PCR was carried out using primers to sequences specific for splice MOs was confirmed by RT–PCR. RNA was extracted from 25 morphants and 25 controls at 48 h.p.f. using the TRIzol (Invitrogen) method of RNA extraction and precipitation. First-strand cDNA was synthesized using random primers (Sigma-Aldrich) and Omniscript transcriptase (QIAGEN), according to the manufacturer’s instructions. Standard PCR was carried out using primers to sequences specific for splice MOs was confirmed by RT–PCR. RNA was extracted from 25 morphants and 25 controls at 48 h.p.f. using the TRIzol (Invitrogen) method of RNA extraction and precipitation. First-strand cDNA was synthesized using random primers (Sigma-Aldrich) and Omniscript transcriptase (QIAGEN), according to the manufacturer’s instructions. Standard PCR was carried out using primers to sequences specific for splice MOs was confirmed by RT–PCR. RNA was extracted from 25 morphants and 25 controls at 48 h.p.f. using the TRIzol (Invitrogen) method of RNA extraction and precipitation. First-strand cDNA was synthesized using random primers (Sigma-Aldrich) and Omniscript transcriptase (QIAGEN), according to the manufacturer’s instructions. Standard PCR was carried out using primers to sequences specific

To visualize head cartilage embryos were allowed to develop to 5 d.p.f, treated with 1-phenyl 2-thiourea from 24 h.p.f. for depigmentation, and then stained with Alcian Blue at 5 d.p.f. in whole mount as described previously (41). For H&E staining embryos, 6 d.p.f were fixed in 4% paraformaldehyde (PFA), dehydrated and embedded in wax. After 10 µm sections were obtained, and stained with classical H&E.

For cilia immunostaining, 24 h.p.f. embryos were dechori-nated and fixed in 4% PFA overnight (O/N) at 4°C washed with dehydrated through 25, 50 and 75%, methanol/PBT (PBS 1% Triton X-100) washes and stored at −20°C. The embryos were rehydrated again 75, 50 and 25% methanol/PBT and digested 10 min with Proteinase K (10 µg/ml in PBT). Blocked with 5% goat serum for 1 h and incubated with mouse monoclonal anti-γ-tubulin (1:200, GTU-88, Sigma) and anti-acetylated tubulin (1:800, 6-11B-1, Sigma) O/N at 4°C Secondary antibodies used were Alexa Fluor goat anti-mouse IgG1 488 and Alexa Fluor donkey anti-mouse
IgG2b 568 594 (Molecular Probes). Nuclei were stained with Hoechst and embryos were mounted in Citofluor. Sox10-gfp (24 h.p.f.) were fixed with 4% PFA, nuclei stained and imaged. Images were captured using a Zeiss 710 Confocal Microscope.

Antibody concentration for the western blots was 1/100 for anti-ocr1l, 1/2000 for anti-γ-tubulin and 1/2500 for the secondary.

Zebrafish mutant neuromast staining

Whole-mount immunofluorescence was performed with mouse acetylated tubulin (Sigma T7451), antibodies at d1:200. Briefly, dechorionated embryos were fixed in 4% PFA/PBS 4°C overnight. Embryos were washed three times in methanol, and were rehydrated in descending concentrations of methanol in PBS-Tween 20 (PBST) solution. Primary antibody was incubated overnight 4°C in 10% FBS/PBST. After six washes of 20 min in PBST, embryos were incubated in fluorescently tagged secondary antibody overnight at 4°C. After three washes of 15 min, embryos were imaged using the Zeiss stereo Lunar V12 fluorescent microscope.

Statistical analysis

Normally distributed data were represented as the mean ± SD of triplicate measurements. Statistical significance of value differences was assessed by applying the t-test. An important part of the biological data analyzed within this work did not follow a normal distribution (failed normality tests). Given their non-parametric nature, data distributions were shown as box plots (constructed with Sigmaplot 11.2) depicting the non-parametric Wilcoxon test. In all cases, experiments were performed at least three times with 20–50 cells analyzed per experiment and per condition unless stated otherwise.

AUTHOR CONTRIBUTIONS


SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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