OFD1 Is a Centrosomal/Basal Body Protein Expressed during Mesenchymal-Epithelial Transition in Human Nephrogenesis

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Abstract. OFD1 is the gene responsible for the oral-facial-digital syndrome type 1, a cause of inherited cystic renal disease. The protein contains an N-terminal LisH motif, considered important in microtubule dynamics, and several putative coiled-coil domains. This study used a combination of microscopic, biochemical, and overexpression approaches to demonstrate that OFD1 protein is a core component of the human centrosome throughout the cell cycle. Using a series of GFP-OFD1 deletion constructs, it was determined that the N-terminus containing the LisH domain is not required for centrosomal localization; however, coiled-coil domains are critical, with at least two being necessary for centrosomal targeting. Importantly, most reported OFD1 mutations are predicted to cause protein truncation with loss of coiled-coil domains, presumably leading to loss of centrosomal localization. Kidney development constitutes a classic model of mesenchymal-epithelial transformation. By immunoprobing human metanephiroi and kidney epithelial lines, it was found that, during acquisition of epithelial polarity, OFD1 became localized to the apical zone of nephron precursor cells and then to basal bodies at the origin of primary cilia in fully differentiated epithelia. These striking patterns of OFD1 localization within cells place the protein at key sites, where it may play roles not only in microtubule organization (centrosomal function) but also in mechanosensation of urine flow (a primary ciliary function).

Oral-facial-digital (OFD) syndromes are a group of developmental disorders that feature malformations of the face, oral cavity, and digits (1,2). OFD type 1 (MIM 311200) is the most common, occurring in 1:250,000 births, and is characterized by X-linked dominant transmission, with prenatal lethality in affected boys (2,3). The clinical spectrum in girls is broad, and penetrance is variable; it includes not only malformations of the oral cavity (cleft palate, lip, and tongue; abnormal dentition; and hamartomas), face (hypertelorism and milia), and digits (syndactyly, brachydactyly, and polydactyly) but also diverse brain malformations, such as agenesis of the corpus callosum (4). A clinical feature that distinguishes OFD1 from other types of OFD syndromes is the occurrence of cystic renal disease; OFD1 patients often develop renal failure, requiring dialysis and renal transplantation in late childhood or adulthood (4–6). The OFD1 locus was mapped to Xp22.2 to 22.3 (7), and mutations in affected individuals were found in Cxorf5, subsequently renamed OFD1 (8–10). This gene codes for a protein of 1011 amino acids, which is expressed in human embryos in organs that are affected by the syndrome (10). An alternative transcript has been detected (10), predicted to code for a protein of 367 amino acids (11). Hereafter, we use the term “OFD1” to refer to the 1011 amino acid protein. OFD1 contains a LisH motif in its N-terminal region, which has been postulated to have a role in microtubule (MT) dynamics (12), in addition to five predicted coiled-coil (CC) domains C-terminal to the LisH motif.

We previously reported preliminary evidence that OFD1 co-localized with γ-tubulin in human undifferentiated embryonic cells (10) and suggested that OFD1 might form part of the centrosome. In the current study, we prove that the OFD1 protein is indeed a bona fide centrosomal component, in part associated with the centrioles. Furthermore, we find that the centrosomal localization is dependent on the CC domains of OFD1, frequently missing in OFD1 patients. Finally, we show that OFD1 becomes located in the basal body, a modified centrosome at the base of the primary cilium, by the end of the mesenchymal-epithelial transition that occurs during human nephrogenesis.

Materials and Methods

Chemicals were obtained from Sigma Aldrich Chemical Company (Poole, UK), unless otherwise stated.

Cell Culture

HEK293 (13) and metanephrine mesenchyme (N70) lines were cultured in DMEM (Invitrogen, Paisley, UK) supplemented with 10% FBS and penicillin-streptomycin (100 IU/ml and 100 μg/ml, respec-
HK2 (ATCC) and renal proximal tubule epithelial cells (RPTEC) (BioWhittaker, Verviers, Belgium) were cultured in DMEM/Hams F12 medium (Invitrogen, San Diego, CA) supplemented with 5% FCS, epidermal growth factor (10 μg/mL), hydrocortisone (36 μg/mL), insulin/transferrin/selenium medium supplement, and antibiotics. All cells were grown at 37°C in a 5% CO₂ atmosphere. For depolymerizing MT, N70 cells were treated with nocodazole (5 μg/mL) at 37°C for 1 h, then kept 30 min on ice before fixing with cold methanol. For inducing ciliogenesis, RPTEC and HK2 cells were grown to confluence on four-well Lab-Tek glass chamber slides (Nunc, Hereford, UK) and then serum-starved for 24 h.

**Immunofluorescence Microscopy**

Centrosomes were isolated from HEK293 and processed as described (14), then imaged after incubation with antibodies against α-tubulin (15) and OFD1, previously validated (10). For immunofluorescence microscopy and confocal imaging of cultured cells, the method described previously (10) was used; these cells were probed with antibodies against β-tubulin (1:200), acetylated α-tubulin (1:1000), γ-tubulin (1:100), and OFD1 (1:200). Two different antisera against a carboxyterminal epitope of OFD1 were used (10), with similar results. Lectin staining was performed by incubating sections for 1 h at room temperature with the following lectins diluted to 2 μg/mL in blocking medium: *Arachis hypogea* Alexa Fluor 594 conjugate (Molecular Probes, Eugene, OR), *Lotus tetragonolobus* FITC conjugate (Vector Laboratories, Peterborough, UK), and lectin from *Dolichos biflorus* FITC conjugate. Fluorescence images in Figures 1 and 2 were captured on a Nikon TE300 inverted widefield epifluorescence microscope (Nikon UK Ltd., Kingston upon Thames, UK) using an ORCA ER CCD camera (Hamamatsu) and Openlab 3.09 software (Improvision) and processed using Adobe Photoshop. Quantitative intensity measurements on HEK293 centrosomes stained with α-tubulin or OFD1 antibodies was performed by measuring the mean pixel intensity within a region of interest of fixed area (30 × 30 pixels) that encompassed the centrosome. This was done using Openlab 3.09 software (Improvision) on live video images while imaging for subsaturating exposure times with a ×60 objective (N.A. 1.4).

**Electron Microscopy**

Cells that were grown on Permanox four-well Lab-Tek chamber slides (Nunc) were fixed for 10 min in freshly prepared paraformaldehyde 2% and 5 min in cold methanol. Immunostaining with OFD1 antisera or preimmune serum was performed as described previously (10), followed by a 1 h incubation with goat anti-rabbit antibody conjugated with 1 nm of gold particles (1:100; BBInternational, Cardiff, UK). Samples were silver-enhanced using the Silver Enhancing Kit (BBInternational), post-fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer and 5 mM CaCl₂ (pH7.4) and stained with 1% aqueous osmium tetroxide for 45 min. Sections were viewed in JEM 1200EX transmission electron microscope and photographed using Kodak EM film.

**Cloning and Transfections**

Fragments of OFD1 cDNA obtained by PCR reaction were cloned into pcDNA3.1 myGFP vector (10), in frame with GFP at the 5’ to generate constructs Δ1, Δ2, Δ4, Δ5, and Δ8 (Figure 3). PCR primers are listed in Table 1. Each forward PCR primer included a BamHI restriction site, and each reverse primer included an XbaI or ApaI site, allowing cloning in the multiple cloning site of the vector. Construct Δ3 (Figure 3) was obtained by digestion of the full-length OFD1 construct with BamHI and XbaI, producing the excision of the 3’ end; after fill-in reaction performed with Klenow polymerase, it was relegated on itself. Construct Δ6 (Figure 3) was produced by deletion of a G in

![Figure 1](image-url). OFD1 in the centrosome. Centrosomes were isolated from HEK293 cells and analyzed by immunofluorescence microscopy (A through I) and Western blotting (J). For immunofluorescence microscopy, centrosomes were stained with antibodies against OFD1 (green; A) and α-tubulin to label the centrioles (red; B). Merged images of centrosome pairs (C through F) show partial co-localization of OFD1 with α-tubulin at centrioles (arrows in C). OFD1 was also detected in an extended region of pericentriolar region (dotted line in C). Centrosomes from the same preparation were stained with mouse (G) and rabbit (H) antibodies against γ-tubulin followed by Alexa-594 goat anti-mouse (red) and Alexa-488 goat anti-rabbit (green) antibodies. Merged images (I) show perfect overlap indicating no microscopic chromatic aberration. Bar = 2 μm. For Western blotting (J), 10 μg of protein samples from total lysates of HEK293 cells (T) and isolated centrosomes (C) were separated by SDS-PAGE and then either Western blotted and probed with antibodies against OFD1, γ-tubulin, and β-actin as indicated or silver-stained. OFD1 and the known centrosomal marker γ-tubulin were detected in total lysates and isolated centrosome preparation, whereas β-actin was barely detected in the centrosome preparation. Molecular weight markers (kD) are indicated.
position 312 from the wild-type construct using Quikchange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA); this deletion produces a frameshift that leads to a stop codon at nucleotide 432. Construct /H9004 (Figure 3) was produced by ligating the PCR product of the 5' region to construct /H9004, both digested with EcoRI, obtaining an in-frame cDNA missing nucleotides 816 to 1097. The two single-nucleotide substitutions were introduced in the OFD1 full-length cDNA using Quikchange XL Site-directed Mutagenesis kit (Stratagene). All of the constructs have been sequenced using ABI Big Dye Terminator Sequencing Kit and ABI 377 Sequencer (Perkin Elmer, Foster City, CA). Restriction enzymes and Klenow polymerase were purchased from New England Biolabs (Hitching, UK). The day before transfection, HEK293 were plated at subconfluent density on four-well Lab-Tek chamber slides. They were incubated for 6 h in 500 μl of serum-free medium that contained 2 μl of Lipofectamine (Invitrogen) and 1μg of DNA per well. After fixation with cold methanol, cells were immunostained for γ-tubulin, detected with a Cy5-conjugated secondary antibody, and analyzed with confocal laser scanning microscope (Leica, Milton Keynes, UK).

Figure 2. OFD1 in the cell cycle. Asynchronous HEK293 cells were processed for indirect immunofluorescence microscopy using anti-OFD1 antisera (green), anti-γ-tubulin antibodies (red) to detect the centrosome, and Hoechst 33258 to stain DNA. For each wavelength, images were captured and processed under identical conditions. OFD1 preimmune serum did not detect centrosomes (A through D), whereas the immune serum stained a large area reminiscent of the PCM in interphase cells (E through H). The field depicted in I through L contains interphase and mitotic cells, and the latter are indicated by an asterisk in their nuclei. In mitotic cells, the OFD1 signal was present but in a smaller, tighter region reminiscent of centrioles (arrows in I and L). Bar = 5 μm. (M) The relative abundance (arbitrary units) of γ-tubulin and OFD1 (measured using two independent antisera, OFD1[a] and OFD1[b]) at interphase centrosomes and metaphase spindle poles was compared. Interphase (I) intensities were quantitated on regions of interest that encompassed the complete centrosome area, whereas mitotic (M) values are given as the sum of the intensities at the two spindle poles. Centrosomes in at least 50 cells were imaged for each condition, and error bars represent SD (P < 0.0001 for OFD1).
Immunohistochemistry

The three metanephroi analyzed derived from terminated normal human fetuses (7, 9, and 12 wk gestational age) collected under research ethical committee permission. Samples were preserved at −80°C in OCT embedding medium (LAMB Laboratory Supplies, Eastbourne, UK). Eight-micrometer-thick cryosections fixed in methanol at −20°C for 10 min were immunostained following the same procedure used for immunocytochemistry, apart from washings, done with PBS 0.01% MgCl₂ and 0.01% CaCl₂, and analyzed by confocal laser scanning microscopy.

Figure 3. Centrosomal targeting of OFD1. (A) Individual GFP-OFD1 fusion proteins and their centrosomal localization (+ [present at the centrosome], +/- [only a subset of transfected cells show centrosomal localization of OFD1], or − [absent from centrosomes]). The yellow ovals indicate LisH motifs; purple boxes indicate the CC domains. (B through D) An example of GFP-OFD1 fusion protein localized to the centrosome (arrows), in this case, a wild-type transfecant is shown, whereas E through G show an example in which the fusion protein fails to localize to the centrosome (arrows in E and G), in this case, a construct Δ5 transfecant. B and E are immunostained with γ-tubulin (red), C and F show GFP-OFD1 green fluorescence, and D and G are merged pictures. Bar = 2 µm.
Western Blotting

Preparation and Western blotting of whole-cell lysates and isolated centrosome preparations were performed as described (16) using anti-OFD1 antisera, β-actin, or γ-tubulin antibodies.

Results

Localization of OFD1 in the Centrosome

Consistent with our previous data on primary kidney cell lines (10), immunofluorescence microscopy with OFD1 antisera revealed a signal that co-localized with the centrosomal protein γ-tubulin in HEK293 cells. We therefore decided to isolate centrosomes from an asynchronous culture of HEK293 cells and probe them for OFD1. With this isolation method, MT are disassembled and centrosomes retain their typical composition of two centrioles surrounded and connected by pericentriolar material (PCM). Immunostaining of isolated centrosomes with γ-tubulin, a major structural protein of the centriole cylinders, and anti-OFD1 antiserum showed that OFD1 was clearly present at both centrioles as well as, more weakly, in an area extending beyond γ-tubulin immunoreactivity, which might represent PCM (Figure 1, A through C). Close inspection of isolated centrosomes revealed that there was not a precise co-localization of γ-tubulin and OFD1 but that OFD1 tended always to overlap with one end of the γ-tubulin staining (Figure 1, C through F). This might suggest that OFD1 is closely associated with the centrioles but enriched toward one end of these cylindrical structures. To rule out the possibility of chromatic aberration, we double-labeled centrosomes with anti-γ-tubulin antibodies raised in two different species, with exact overlap of the two fluorescence signals (Figure 1, G through I). Western blotting with OFD1 antibodies revealed the presence of a doublet at ~110 kD in both total HEK293 lysates and isolated centrosomes (Figure 1J). This corresponds to the predicted mass of OFD1 and falls in line with previous observations of OFD1 by Western blot (10).

Next, we performed immunoelectron microscopy (immuno-EM) on a human metanephric mesenchyme cell line (N70). We previously showed that this cell line expresses OFD1 as assessed by immunoblotting, and OFD1 co-localized with γ-tubulin in immunocytochemistry (10). Centrosomes were clearly identified in sections of 32 cells, as assessed by the morphologic detection of centrioles, and in 29 cells these were decorated with gold particles. In most cases, gold particles were closely associated with the centrioles themselves (Figure 4, A

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Table 1. PCR primer for OFD1 constructs

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<td>Δ2</td>
<td>nt 1–1888</td>
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Figure 4. Electron microscopy (EM) of N70 cells with immunogold labeling for OFD1. Human metanephric mesenchyme cells were fixed and processed for immuno-EM. Gold particles were closely associated with the centrioles themselves (asterisks, A and B). Some gold particle labeling was also detected in the vicinity of the centrioles (within 500 nm), presumably corresponding to pericentriolar material (PCM; C and D). Bar = 500 nm.
and B). Some gold particle labeling was also detected in the vicinity of the centrioles (within 500 nm), presumably corresponding to PCM (Figure 4, C and D). These EM data therefore correspond very closely to the immunofluorescence observations made on isolated centrosomes. Next, N70 metanephric mesenchyme cells were incubated on ice in the presence of nocodazole to depolymerize the MT network (17). Control (Figure 5A) and treated cells (Figure 5B) were probed with β-tubulin antibody to visualize MT and OFD1 antisera. It was clear that the normal radial arrangement of MT, apparent in controls, was abolished in treated cells. A discrete OFD1 signal was seen in the microtubule organizing center (MTOC) of control cells, and after MT disassembly, a comparable OFD1 signal was seen. Hence, the localization of OFD1 with centrosomes was not dependent on the presence of polymerized MT. Collectively, these data demonstrate that OFD1 is a genuine core component of the centrosome in human embryonic kidney cells.

**OFD1 Associates with the Centrosome throughout the Cell Cycle**

To determine whether there is any change in association of OFD1 with the centrosome through the cell cycle, we next used OFD1 antisera to detect OFD1 in asynchronous HEK293 cells. Preimmune OFD1 antisera showed no signal (Figure 2, A through D). Using OFD1 immune sera, all cells in interphase displayed a prominent OFD1 signal usually as one large dot, often near the nucleus, that co-localized with γ-tubulin immunostaining; no obvious difference in abundance was seen between interphase cells, suggesting that there is no change in centrosomal abundance between cells in G1, S, or G2 phase (Figures 2, E through H, and 6A). During mitosis, OFD1 staining appeared as a smaller dot (Figure 2, I through L). After immunofluorescence staining of HEK293 cells, the relative abundance of γ-tubulin and OFD1 at interphase centrosomes and metaphase spindle poles was compared. Quantitative analysis indicated a decrease of ~50% of OFD1 in metaphase cells compared with interphase (Figure 2M); as expected, the overall abundance of γ-tubulin increases at centrosomes in mitosis (18). By analyzing all mitotic stages, from prophase to telophase, OFD1 was always detectable as discrete dots (Figure 6, B through F); co-labeling with an antibody against β-tubulin showed that at the stages when the spindle is present, OFD1 is located at the poles (data not shown).

**Centrosomal Targeting of OFD1 Depends on CC Motifs**

To investigate which regions of OFD1 are required for centrosomal localization, we generated a series of constructs that represent full-length wild-type OFD1, truncated OFD1, or full-length OFD1 with missense mutations found in individuals who are affected by the syndrome (Figure 3A). In these constructs, OFD1 was fused at its N-terminus to GFP. Constructs were transiently transfected into HEK293 cells, and the localization of the resulting GFP-OFD1 fusion protein was compared with that of the centrosomal marker γ-tubulin. As expected, full-length recombinant OFD1 localized to the centrosome, providing further support for the localization of endogenous OFD1 to the centrosome. C-terminal deletion constructs missing regions encoding domains CC5, CC4/5, and CC3 to 5 (Δ1, Δ2, and Δ3) also localized to the centrosome. In these transfected cells, we also observed a variable amount of cytoplasmic fluorescence, suggesting that overexpressed protein not accommodated at the centrosome was in the cytoplasm, as noted for other centrosomal proteins expressed in mammalian cells (19). In constructs that retained the N-terminal LisH motif but that lacked all five CC domains (Δ5 and Δ6), no specific centrosomal localization was observed, whereas the construct Δ4, comprising the LisH domain together with CC1, localized to the centrosome in approximately half of the transfected cells. These results led us to hypothesize that the region encompassing CC1 and, perhaps, CC2 might be important for OFD1 centrosomal localization. We therefore generated two more constructs, the first missing the sequence coding for just CC1 and the second lacking the LisH domain as well as CC1 and CC2, respectively constructs Δ7 and Δ8 in Figure 3A. Surprising, both of these constructs localized to the centrosome, suggesting that OFD1 is not localized to the centrosome by one particular region but rather that a combination of CC domains is probably important for this process. We then introduced into the GFP-OFD1 full-length construct two different missense mutations. The first is a C/T transition that leads to the substitution of a serine residue with a phenylalanine in the LisH domain. This models a de novo mutation in

![Figure 5](https://example.com/figure5.png)

*Figure 5.* Treatment of N70 cells with nocodazole. Control (A) and treated cells (B) were probed with β-tubulin antibody in red, to visualize microtubules (MT), and OFD1 antisera in green. Note the normal radial arrangement of the MT in A, whereas in B, no MT pattern can be discerned. A discrete OFD1 signal is seen in the microtubule organizing center region in control cells (arrows); after MT disassembly, a comparable OFD1 signal was seen. n, nucleus. Bar = 2 μm.
an individual with OFD1 syndrome (family 4 in reference 10). The second mutation is an A/C transition that leads to a change from serine to arginine in the second CC domain of the protein. This is an inherited missense mutation found in an OFD1 kindred (family 1.1 in reference 8). Both constructs (mis 1 and mis 2; Figure 3A) localized to the centrosome, indicating that the defect in these patients was not the result of mislocalization of the protein at the centrosome. Figure 3B, A through C, shows an example of transfection in which the green fluorescence fusion protein is targeted to the centrosome (wild-type transfectant), whereas Figure 3B, D through F, shows an example of impaired localization (Δ5 transfectant).

**OFD1 Localization during Kidney Development**

Previously, we immunolocalized OFD1 in human first-trimester metanephric mesenchyme using paraformaldehyde-fixed paraffin-embedded tissues (10). To optimize the quality of the immunostaining, we repeated these histologic studies on frozen sections. Using this protocol, we noted widespread immunoreactivity in a range of structures, and this signal was abolished when the primary antibody was either substituted with preimmune serum or preabsorbed with the immunizing peptide (Figure 7, A through C). In metanephros of 7 and 8 wk of gestational age, we observed OFD1 expression in peripheral and condensing mesenchyme as a single dot co-localizing with the centrosome (Figure 7, D through F). In vesicles, which represent an early stage of mesenchymal to epithelial transition, OFD1 immunolocalized to the apical region of the cell, between the nucleus and the lumen (Figure 7, G through I). γ-Tubulin, a marker for centrosomes and noncentrosomal MTOC in polarized epithelia, co-localized with OFD1 in these nephron precursors, in a pattern of multiple dots. A broadly comparable pattern is seen in the stalk of ureteric bud branches, in the same specimens (Figure 7, J through L). We then examined a more mature sample (12 wk) and to specify the epithelial structures used fluorescence lectins (Figure 8). This analysis showed that OFD1 immunolocalized in distal tubules (Figure 8, A and B), collecting ducts (Figure 8, C and D), and proximal tubules (Figure 8, E through H).

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**Figure 6.** OFD1 in mitotic cells. Immunofluorescence microscopy was performed on HEK293 cells in interphase (A) and different stages of mitosis (B through F). Cells in A through F are stained with OFD1 antiserum in green and propidium iodide in red to show condensation of chromosomes. OFD1 signal at the spindle poles is indicated by arrows. Bar = 2 μm.
Figure 7. OFD1 in mesenchyme and epithelia. Sections from a metanephros of 7 wk gestational age were stained with propidium iodide in red (A) or OFD1 antiserum (B) or preimmune serum (C) in green. Metanephric structures such as ureteric bud (u), vesicles (v), mesenchyme (m), and stromal cells (s) are visible. (D through L) Greater detail of OFD1 immunostaining (D, G, and J), γ-tubulin immunostaining (E, H, and I), and merged pictures (F, I, and L) of those structures. In the mesenchyme, OFD1 signal co-localizes with γ-tubulin immunostaining (arrows in D, E, and F), as a single dot per cell. In vesicles (outlined by the dotted line in G, H, and I), OFD1 co-localizes with γ-tubulin immunostaining as a punctate pattern in the apical region of the forming epithelium. In a ureteric bud branch (outlined by the dotted line in J, K, and L), OFD1 co-localizes with γ-tubulin staining in a dot-like pattern and is also detected as a diffuse signal in the apical portion of the epithelium. The lumen of the epithelial structures is indicated by l (G through L). In J, crosses indicate individual cell bodies. Bar = 50 μm in A through C, and 10 μm in D through L.
Figure 8. Lectin labeling. A 12-wk metanephros was probed with *Arachis hypogea* (A and B), *Dolichos biflorus*, (C and D), and *Lotus tetragonolobus* fluorescently labeled lectins (E through H). Double labeling with lectins and OFD1 antiserum shows presence of OFD1 in the apical portion of epithelial cells in distal tubules (A) and the collecting duct lineage (C); E shows a tubule labeled with the proximal tubule lectin, and in F, it is apparent that the same tubule expresses OFD1 in a punctate pattern. G shows the merged images of E and F. When preimmune serum was used, no apical signal was detected (B, D, and H). Note that in A and B, the red and green colors have been reversed; therefore, all of the lectin staining appears in green and OFD1 signal appears in red. Bar = 10 μm.
**OFD1 Localizes to the Basal Body of the Primary Cilium**

To assess whether OFD1, like several proteins involved in polycystic kidney disease (PKD) and other inherited cystic diseases, is localized to primary cilia, we cultured human nonimmortalized proximal tubule epithelial cells (RPTEC) under conditions that we found to promote ciliogenesis. In cells that possess a primary cilium, the mother and daughter centrioles are equivalent to and often described as basal body structures. In all cells examined, OFD1 was detected at the basal bodies; in rare instances (<10% cells), OFD1 was also immunolocalized to the length of the primary cilium itself, with a discontinuous pattern (Figure 9, A through C). We also examined another human proximal tubule cell line, HK2 (20), in conditions of ciliogenesis and found that OFD1 also immunolocalized to the basal bodies (data not shown). Immunohistochemistry of a metanephros of 12 wk of age showed that OFD1 was concentrated in the basal body of ciliated tubular cells (Figure 9, D through F). In sections that were stained with preimmune serum, no signal was detected in the basal bodies (Figure 9, H and I). Thus, OFD1 is a core component not only of centrosomes in cycling cells but also of basal bodies and possibly primary cilia in postmitotic differentiated cells.

**Discussion**

**OFD1 is a Centrosomal Protein**

Our new data demonstrate that OFD1 is a *bona fide* component of human kidney centrosomes. This supports our preliminary immunocytochemistry results on embryonic kidney cells (10). The centrosome is a nonmembranous organelle that acts as the primary site of MT nucleation in the vast majority of vertebrate cells (21). Its localization at spindle poles dictates the polarity and axial positioning of the mitotic spindle on which chromosomes are segregated during cell division. Recent experiments defined additional roles for this organelle in cytokinesis and at G1/S transition (22,23). The centrosome consists of a centriolar doublet, each composed of cylinders of nine triplets of MT, surrounded by a protein matrix called the PCM. Within each centriolar doublet, there exists a mother centriole, characterized by distal and subdistal appendages, and a daughter centriole, lacking those appendages. Of those centrosomal proteins studied so far, some have been localized to

*Figure 9. OFD1 in primary cilia.* Renal proximal tubule epithelial cells (RPTEC) were stained with OFD1 antiserum (A) and with acetylated α-tubulin to label the primary cilium (B). The merged picture (C) shows localization of OFD1 signal in the basal body of the cilium (arrow) and some dots in the stalk of the cilium as well (arrowheads). Sections from a metanephros of 12 wk gestational age immunolabeled with OFD1 antiserum (D), acetylated α-tubulin (E), and merged picture (F) show that OFD1 is concentrated in the basal bodies of cilia in renal tubules. No signal is detected in the basal bodies when preimmune serum is used instead of OFD1 antiserum (G through I). *Lumen of the tubules. Bar = 0.5 μm in A through C, and 7 μm in D through I.*
both centrioles (e.g., centrin, C-Nap1), others localize predominantly to the mother centrioles (e.g., ninein, α-tubulin), and others localize mainly to the PCM (e.g., γ-tubulin, pericentrin) (21,24). The PCM, containing γ-tubulin ring-like complexes, is the primary site of MT nucleation (25), whereas the mother centriole appendages are thought to act as sites of MT anchoring (24). Immunofluorescence microscopy of isolated centrosomes, together with immuno-EM in undifferentiated human embryonic kidney cells, demonstrated that OFD1 protein associates closely with both mother and daughter centrioles as well as to the PCM. Furthermore, in whole cells that were treated to depolymerize the MT network and in preparations of isolated centrosomes, OFD1 remained tightly associated with centrosomes. This confirms that OFD1 is a genuine core component of the centrosome and not simply concentrated at the minus ends of MT. Our current data do not exclude the possibility that OFD1 is also located at noncentrosomal sites in HEK293 and metanephric mesenchymal cells, as has been reported for other “archetypal” centrosomal molecules, such as γ-tubulin (18).

The composition of the centrosome changes during the cell cycle as the organelle undergoes a semiconservative replication of centrioles in S and G2 phases and recruitment of additional MT nucleating capacity just before mitosis (26). As well as detecting OFD1 in all interphase cells examined by immunocytochemistry, OFD1 was precisely localized on centrosomes at all stages of mitosis from prophase to telophase, when OFD1 localized to the spindle poles. However, there was a significant decrease in the amount of OFD1 present at the centrosome during mitosis. Other centrosomal proteins are known to be either decreased or completely absent from mitotic spindle poles. One example is C-Nap1, which is displaced from the spindle poles during mitosis, allowing splitting of the mother and daughter centrioles (27). Another example is Nlp, levels of which must fall during mitosis to allow the construction of the mitotic scaffold (28). The decrease of OFD1 that we have observed during mitosis would be consistent with its having a similar role to either C-Nap1 or Nlp. It is interesting that the localization of both of these proteins is regulated through phosphorylation by centrosomal protein kinases; it will be interesting to determine whether OFD1 is similarly regulated.

**OFD1 Targeting to the Centrosome**

De Conciliis et al. (11) predicted that the OFD1 protein contained several heptad repeats, characteristic of CC. Such domains mediate protein–protein interaction, including homodimerization (29), and are commonly found in centrosomal proteins [e.g., C-Nap1, pericentrin, ninein (19,30)]. Franco et al. (31) presented preliminary evidence that OFD1 homodimerizes through a central region where most of the CC are located. In the current study, we began to address which regions of the protein might be important for centrosomal localization. By transfecting a series of GFP-OFD1 constructs into embryonic kidney cells, we determined that regions of the protein encompassing CC were critical, with at least two apparently necessary for normal localization. However, no one single region of OFD1 seemed to be solely responsible, as constructs that include CC1 and CC2 only or CC3, CC4, and CC5 only were capable of localizing to the centrosome. Further experiments will be required to determine whether the CC domains themselves or nearby regions are critical for centrosomal localization. Most reported OFD1 mutations insert a premature stop codon in the open reading frame (8–10). These transcripts might undergo nonsense-mediated decay (32), but if they were to be translated, then the proteins would lack a variable number of CC. Indeed, in some of these cases, all five CC would be deleted, and one would predict that the mutant protein would not localize to the centrosome. However, other reported OFD1 mutations would lead to the loss of only two or three CC; our transfection results suggest that these proteins may still localize to the centrosome, perhaps suggesting that these parts of the molecule have other, as yet unknown, functions. Furthermore, the GFP-OFD1 construct that codes for the disease causing S435R substitution in CC2 also localizes to the centrosome, again suggesting that a localization defect is unlikely to be the cause of disease in this patient.

The N-terminal domain of OFD1 contains a LisH motif (12). This motif appears in other proteins mutated in human congenital syndromes: The LIS1 protein, mutated in Miller-Dieker lissencephaly, a disease with neural migration defects, and in the protein encoded by Treacle, a gene mutated in Treacher Collins syndrome, an autosomal dominant disorder with craniofacial malformations and hearing loss (12). When we deleted the first 104 amino acids that encompass the OFD1 LisH domain but retain all of the CC, we observed normal centrosomal localization of the GFP-OFD1 construct. Furthermore, a construct that harbors a reported missense mutation in the LisH motif, changing an amino acid conserved in human, mouse, and Xenopus (33), also localized to the centrosome. Emes and Ponting (12) suggested several functions for this motif; one of them is MT binding, and another is an ATPase activity, which might disassemble MT. Our observations that in a subfraction of epithelial cells OFD1 localized to the primary cilium and the EM data showing close association with the centrioles suggest that OFD1 might directly bind MT, and perhaps the LisH motif could be involved in this interaction.

**OFD1 Expression during Mesenchymal-Epithelial Transition**

Most differentiated epithelia contain a specialized organelle called a primary cilium. It consists of a protuberance of the apical plasma membrane that ensheathes a rod-like axoneme, composed of nine MT doublets (34). During the differentiation of epithelial cells, from nonpolarized precursors, major rearrangements occur in the cytoskeleton. First, MT are released from the centrosome and align in a longitudinal manner, with their minus ends captured by proteins such as γ-tubulin and ninein, as part of noncentrosomal MTOC, in the apical compartment of the cell (35,36). Second, the centrosome itself takes up a position between the nucleus and the apical plasma membrane, and the distal end of the mother centriole gives rise to the axoneme of the primary cilium (34). Kidney development constitutes a classic model of mesenchymal-epithelial transformation. In the first-trimester human metanephros, at any single time, there coexists a spectrum of cells from undif-
ferentiated mesenchyme, to polarizing nephron precursors, as well as ureteric bud branches, which represent relatively mature epithelial tubules. By immunoprobeing human metanepho
tipe at a stage when nephrons have yet to fully form, we found that, during acquisition of epithelial polarity, OFD1 became localized to the apical zone of nephron precursor cells. By using a panel of lectins to mark more mature tubules, we proved that OFD1 is present in tubules in both the nephron and ureteric bud lineage; in proximal tubules, OFD1 signal appears as a punctate pattern, probably correlated with basal bodies; indeed, we confirmed the presence of OFD1 in basal bodies in ciliated human proximal tubule kidney epithelial lines. In other kidney tubules visualized by immunohistochemistry, OFD1 appeared as a more diffuse apical pattern. We speculate that this appearance may represent OFD1 associated with noncentrosomal MTOC (35,36), but this contention would need to be supported by further experiments, e.g., double immunostaining for OFD1 and ninein.

Mutations in genes coding for proteins localized in the primary cilium are often associated with human inherited cystic diseases (37–39): they include the proteins implicated in autosomal dominant PKD (polycystin 1 and 2), autosomal recessive PKD (fibrocystin), and several types of nephronophthisis (e.g., nephrocystin, inversin). In addition, polaris and cystin are mutated in mouse models of PKD. All of these proteins are immunolocalized to the stalk of the primary cilium; furthermore, polycystin 1 (40) and inversin (41,42) immunolocalize to basal bodies. Although we detected relatively low levels of OFD1 in the stalk of primary cilia, the predominant signal was in the basal body. It has already been proved that the polycystins are plasma membrane–associated proteins, which form calcium channels activated by mechanical distortion of the cilium (40). Conversely, polaris is part of the intraflagellar transport system within the cilia, and affected animals have stunted cilia (43,44). On the basis of the prominent basal body location of OFD1, we speculate that this protein is involved in the biogenesis of primary cilia, and in the future, could be explored either by knocking down OFD1 in cells in culture or by looking at the ultrastructure of cells from OFD1 patients. Furthermore, it would be important to determine whether there is any direct interaction between OFD1 and these other proteins, and it is worth noting that both polycystins contain CC, which mediate their interaction (45).

Acknowledgments
The work was supported by Action Medical Research (S.A.F., L.R., S.M., and A.S.W.), the Kidney Research Aid Fund, Wellcome Trust (A.M.F.), and Association of International Cancer Research (A.M.F.). A.M.F. is a Lister Institute Research Fellow.

The content of this paper was presented as a poster at the American Society of Nephrology Conference (Renal Week, November 2003), San Diego, CA.

We thank Kerrie Venner, for technical help concerning the EM, and the MRC/Wellcome Trust Human Embryo Bank, Institute of Child Health, London, for provision of tissue samples.

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