Functional Characterization of the OFD1 Protein Reveals a Nuclear Localization and Physical Interaction with Subunits of a Chromatin Remodeling Complex

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Oral-facial-digital (OFD) type I syndrome is an X-linked dominant disease (MIM311200) characterized by malformations of oral cavity, face, and digits and by cystic kidneys. We previously identified *OFD1*, the gene responsible for this disorder, which encodes for a centrosomal protein with an unknown function. We now report that OFD1 localizes both to the primary cilium and to the nucleus. Moreover, we demonstrate that the OFD1 protein is able to self-associate and that this interaction is mediated by its coiled-coil rich region. Interestingly, we identify an OFD1-interacting protein RuvB1, a protein belonging to the AAA⁺-family of ATPases, which has been recently associated to cystic kidney in zebrafish and to ciliary assembly and function in *Chlamydomonas reinhardtii*. We also provide experimental evidence that OFD1, together with RuvB1, is able to coimmunoprecipitate with subunits of the human TIP60 histone acetyltransferase (HAT) multisubunit complex. On the basis of these results, we hypothesize that OFD1 may be part of a multi-protein complex and could play different biological functions in the centrosome-primary cilium organelles as well as in the nuclear compartment.

INTRODUCTION

Oral-facial-digital syndromes (OFDs) are a heterogeneous group of developmental disorders for which nine different forms have been described. OFD type I (MIM311200) presents an X-linked dominant pattern of inheritance with lethality in males (Doege *et al.*, 1964; Wettke Schäfer and Kantner, 1983). Affected females have malformations of oral cavity (cleft palate, lip and tongue, abnormal dentition, and hamartomas), face (hypertelorism and milia), and digits (syndactyly, brachydactyly, and polydactyly), with a highly variable severity even within the same family. Involvement of the CNS includes mental retardation, hydrocephalus, cerebellar anomalies, porencephaly, and agenesis of the corpus callosum (Towfighi *et al.*, 1985; Odent *et al.*, 1998). All these clinical features overlap with those reported in the other forms of OFDs. Among these, type 1 can be easily distinguished for the presence of cystic kidneys, with reports of patients in which the renal involvement completely dominates the clinical course of the disease (Connacher *et al.*, 1987; Feather *et al.*, 1997). We identified the gene, named *OFD1*, responsible for this genetic disorder, we showed that it is expressed during development and in adult tissues, in all the structures affected in this syndrome.

The *OFD1* gene encodes a 1011-amino acid protein that shares no sequence homologies with proteins having known function. Five predicted coiled coil domains (hereafter CC) occupy almost the entire length of the molecule (de Conciliis *et al.*, 1998), whereas the N-terminal region shares a Lis1 homology motif (LisH) with over 100 eukaryotic intracellular proteins (Emes and Ponting, 2001). Data from the literature indicate that the alpha-helical CC, despite its simplicity, is a highly versatile folding motif found in proteins with different functions and is described to mediate subunit oligomerization (Burkhard *et al.*, 2001). The biological function of the LisH motif is still unknown, although it has been postulated to be involved in cell migration, nucleokinesis, and chromosome segregation (Emes and Ponting, 2001). More recent data suggested that this motif could mediate protein dimerization (Kim *et al.*, 2004) and transcriptional activation (Wei *et al.*, 2003).

We recently described knockout animals that reproduce the main features of this genetic condition, and we demonstrated that *Ofd1* is required for primary cilia formation and left-right axis specification (Ferrante *et al.*, 2006). OFD1 co-localizes with γ -tubulin and is a centrosome-associated protein (Romio *et al.*, 2003), and more interestingly, it has been described to localize at primary cilia in fully differentiated renal epithelial cells (Romio *et al.*, 2004). Although the specific biochemical function is still unknown, the intracellular localization of OFD1 correlates well with the phenotype described in knockout animals. We now report further data that validate the localization of OFD1 to the primary cilium, and for the first time, we describe the presence of OFD1 in the nucleus. We also provide evidence that OFD1 is able to self-associate through its CC-rich region. Finally, we report the identification of an OFD1 molecular partner, RuvB1, a

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protein belonging to the AAA⁺-family of ATPases, involved in transcriptional regulation and cell division and recently associated to cystic kidneys. RuvB1 is part of the human histone acetyltransferase (HAT) multisubunit complex known as TIP60 complex, and we provide data indicating that OFD1 is part of the same complex.

MATERIALS AND METHODS

Anti-OFD1 Antibodies

Two rabbit polyclonal anti-OFD1 antisera, named anti-OFD1 Cter and anti-OFD1 cent, were produced (Neosystem, Strasbourg, France). The following synthesized oligopeptides at the C-terminal and central regions of the OFD1 protein were used as antigen: KVESLTGFSHEELDDSW (residues 996-1011) and RTNRLIEDERKNKEK (residues 351-364), respectively. The two antisera, as well as the preimmune serum, were used after purification by affinity chromatography with a protein A-Sepharose matrix (Amersham Pharmacia, Piscataway, NJ).

Constructs for Expression in Mammalian Cells

The coding region for *OFD1* was amplified by PCR using a plasmid containing the full-length cDNA for *OFD1* as template and specific primers designed on the available sequence. The resulting fragment was cloned into the pcDNA₃-MycGFP (Invitrogen, Carlsbad, CA) and pcMV-Flag (Sigma, Milan, Italy) mammalian expression vectors, in frame with the MycGFP and Flag tags at its N-terminus. To introduce the 1757delG mutation into the MycGFP-OFD1 construct, in vitro mutagenesis was performed using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, CA), according to the manufacturer's instructions. The presence of the point mutation was then confirmed by DNA sequencing. The following primer was used for site-directed mutagenesis: 5'-TGCAATGGTGAGATAATGGGGATTCTTGAACA-3'. For the hemagglutinin (HA)-RuvB1 and MycGFP-RuvB1 constructs, the coding region of RuvB1 was obtained as reported in *Materials and Methods* describing the yeast two-hybrid system (below), and the resulting fragments were cloned in pcDNA3-HA or pcDNA3-MycGFP vectors, in-frame with the HA or MycGFP tags at their N-terminus.

Cell Culture, Cell Transfection, and Immunofluorescence

Monkey kidney Cos-7 (Cos-7) and Madin-Darby canine kidney (MDCK) cells were maintained in exponential growth in DMEM (DMEM, HyClone Laboratories, Logan UT) containing 10% fetal bovine serum (HyClone Laboratories), 100 Units/ml penicillin, and 100 µg/ml streptomycin at 37°C with 5% CO₂. All constructs were transfected using Polyfect (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. For immunofluorescence experiments, Cos-7 and MDCK cells were grown on coverslips and kept at confluence. MDCK were cultured for at least 5 d before analysis, to promote ciliogenesis (Ward *et al.*, 2003). Cells were examined with an Axioplan microscope (Zeiss, Thornwood, NY) equipped with an Axiocam CCD camera and Axiovision digital imaging software (Zeiss). Alternatively, cells were analyzed with a confocal microscope (Leica, Deerfield, IL).

The polyclonal anti-OFD1 Cter and anti-OFD1 cent antibodies and the preimmune serum were from Neosystem and the anti-acetylated α -tubulin (1:1000) antibody was from Sigma (T6793). All the secondary antibodies for immunofluorescence were from Dako (Carpinteria, CA, 1:200).

Nucleus-Cytoplasm Fractionation

Cos-7 cells, 3×10^6 , were lysed in buffer containing 10 mM Tris HCl, pH 7.5, and Triton 1%, supplemented with 1 mM protease inhibitor cocktail (Sigma), and a small amount was kept in ice (total cell lysate). After centrifugation, the pellet was resuspended in PTG buffer (10 mM Tris HCl, pH 7.4, 2 mM dithiothreitol [DTT], 10% glycerol, 1 mM MgCl₂), supplemented with protease inhibitors. After 10 min in ice, NP40 0.5% was added and the sample was gently mixed at RT for 3 min. The sample was then centrifuged at $1200 \times g$ for 4°C for 5 min; the supernatant, corresponding to the cytosolic fraction, was conserved in ice, and the pellet, corresponding to the nuclear fraction, was resuspended in the lysis buffer. Protein concentration was measured by Bio-Rad Bradford assay (Richmond, CA) and the same amount of protein (20 µg) from nuclear and cytosolic fraction and from total cell lysate was loaded. For Western blot, the purified anti-OFD1 Cter antibody was used at 1:250, and monoclonal anti- β -tubulin (Sigma) and polyclonal anti-acetyl-Histone H4 (Upstate Biotechnology, Lake Placid, NY) were used, both at 1:2000.

Immunoprecipitation and Western Blotting

To coimmunoprecipitate OFD1, 1×10^6 Cos-7 cells were cotransfected with MycGFP-OFD1 and Flag-OFD1-expressing vectors or were transfected with only one of the two vectors. After 24 h of expression, the cells were lysed in a buffer containing 10 mM Tris HCl, pH 7.5, NaCl 150 mM, and Triton 1%, supplemented with protease inhibitor. The lysates precleared with protein

A-Sepharose beads (Sigma) were supplemented with the appropriate primary antibody. Polyclonal anti-Myc (Upstate Biotechnology) and monoclonal anti-Flag M2 were used at 1:100 for immunoprecipitation (IP) and at 1:500 for immunoblot (IB).

To demonstrate the interaction of OFD1 with the overexpressed RuvB1, Cos-7 cells were transfected with the HA-RuvB1-expressing vector and, as a control, with the pcDNA3-HA empty vector. After 12 h of expression the precleared cells lysates were immunoprecipitated with a monoclonal anti-HA antibody (Boehringer Mannheim, Indianapolis, IN; 1:50). For the same purpose, Cos-7 cells transfected with the MycGFP-RuvB1-expressing vector or the empty vector were immunoprecipitated with the anti-OFD1 C-ter antibody (1:60). To demonstrate the interaction of OFD1 with the endogenous RuvB1 and with the TIP60 subunits, lysates from untransfected MDCK cells were used. The precleared lysates were supplemented with the polyclonal anti-OFD1 Cter antibody (1:60), and the immunocomplexes were eluted with the OFD1 peptide KVESLTGFSHEELDDSW. For the opposite experiment, the anti-TIP60 was used 1:100 for IP. In all these experiments, the cells were lysed in a buffer containing 50 mM Tris, pH 7.9, 0.1% Tween 20, 1% Triton, 150 mM NaCl, 5 mM MgCl₂, 10% glycerol, and 5 mM DTT, supplemented with protease inhibitors.

For Western blot analysis antibodies were used at the following concentrations: anti-OFD1 Cter 1:100, anti-RuvB1 1:500, anti-DMAP (Affinity BioReagents, Golden, CO) 1:500; anti-TIP60 (Upstate Biotechnology 1:400); anti-TRRAP 1:200 and anti-RuvB1 1:500. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Amersham Pharmacia (1:3000–4000).

Yeast Two-Hybrid Screening

The two-hybrid screening was performed as previously described (Gyuris *et al.*, 1993). Full-length *OFD1* cloned in the pEG202 vector fused to the LexA DNA-binding domain was used as bait. In the screening, LexAop-LEU2 and LexAop-LacZ genes were used as reporter genes. The bait was transformed into the yeast strain EGY48 that was subsequently transformed with a human HeLa cDNA library cloned into pJG4-5, containing the B42 activation domain. Transformants (0.7×10^6 independent clones) were seeded on plates containing either X-gal or lacking leucine to select positive clones. Interaction mating assay was performed using the same system and two different mating types (EGY48 MAT α and EGY42 MAT α) as described (Finley and Brent, 1994). For homotypic interaction experiments, the fragments OFD1a (residues 1-276), OFD1b (residues 277-663), and OFD1c (residues 664-1011), as well as the 1757delG mutant were cloned in both the yeast pEG202 and pJG4-5 vectors, in frame with LexA and B42 domains. The cDNA corresponding to the coding region of *RuvB1* was obtained by PCR amplification, using IMAGE clone BM467954 as template and specific primers designed on an available sequence. For hetero-interaction experiments, the fragments obtained were cloned in the yeast pEG202 and pJG4-5 vectors.

Glutathione S-transferase Pulldown Assays

The coding regions of *RuvB1*, *OFD1* full-length and of *OFD1a*, b, c, d (residues 58-608) fragments were cloned in the pGEX-4T-1 bacterial expression vector (Pharmacia). Glutathione S-transferase (GST) fusion proteins were produced in the bacterial strain BL21 (DE3) (Stratagene) by inducing protein expression with isopropyl-1-thio- β -D-galactopyranoside (0.2 mM) for 3–4 h at 37°C. After lysis of bacteria, GST fusion proteins were subsequently captured on glutathione-agarose beads (Amersham Pharmacia). For GST pulldown assay, 1×10^6 Cos-7 cells transfected with MycGFP-OFD1 wild type or mutant forms were lysed in the buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 5 mM DTT, 1 mM EDTA, and 1% Triton) supplemented with protease inhibitors. For Western blot, polyclonal anti-Myc (Upstate Biotechnology) was used 1:500. As a control, in each experiment the lysate was also tested with GST alone.

RESULTS

OFD1 Localizes at Primary Cilium and at Nucleus

We first investigated the cellular localization of endogenous OFD1 protein by confocal microscopy and immunofluorescence. For these experiments we used a polyclonal anti-OFD1 antibody (anti-OFD1 Cter) demonstrated to specifically recognize the endogenous OFD1 protein (Ferrante *et al.*, 2006). To evaluate the localization of the OFD1 protein with respect to the primary cilium, MDCK cells were cultured under conditions that promote ciliogenesis. In confocal microscopy experiments, the anti-OFD1 antibody staining showed a signal that localizes at the base of the primary cilium stained with acetylated α -tubulin, indicating a clear localization of the endogenous OFD1 protein at the basal body (Figure 1, A–C). OFD1 was never detected in the

Figure 1. Subcellular localization of the endogenous OFD1 protein. Localization at primary cilium was investigated by confocal microscopy in MDCK cells. (A) Staining with the anti-acetylated α -tubulin. (B) Staining with the anti-OFD1 C-ter antibody. OFD1 localizes at the basal body of the primary cilium as the merge in C shows. In Cos-7 cells studied by immunofluorescence microscopy, the anti-OFD1 detects also a diffuse signal in the nucleoplasm (E), which disappears when using the preimmune (G). Nuclei are stained with DAPI (D). (H) Nuclear and cytoplasmic fractions from whole Cos-7 cell extracts. Immunoblotting with anti-OFD1 C-ter antibody shows that the endogenous OFD1 is present in both cellular compartments. The same amount of protein from the two compartments and from whole cell was loaded. To verify the purity of the extracts, the samples were immunoblotted with anti- β -tubulin and anti-acetyl-Histone H4.

axoneme, and the signal seemed to be restricted to the basal body of the primary cilium.

In Cos-7 cells, staining with the anti-OFD1 antibody shows a diffuse localization of the protein in the nucleoplasm, in addition to the known centrosome localization (Figure 1, D–F). The nuclear signal disappeared when the preimmune serum was used (Figure 1G). A similar nuclear signal was observed in HeLa and MDCK cells, and the same result was obtained with a different antibody directed against the central portion of the OFD1 protein (anti-OFD1 cent; data not shown). To further validate the presence of endogenous OFD1 in the nucleus, we performed a biochemical fractionation of the nuclear and cytosolic compartments. Nuclear and cytosolic extracts from Cos-7 cells were analyzed by Western blot with the anti-OFD1 cent antibody and a specific signal was observed in both compartments. The same quantity of protein extracts was loaded in all lanes. Consistent with the evidence that OFD1 cytosolic localization is restricted to the centrosome-primary cilium, the band corresponding to the cytosolic fraction was weaker than the one corresponding to the nuclear fraction (Figure 1H).

OFD1 Interacts through its CC-rich Region

The OFD1 protein has two main structural characteristics, the CC domains and the LisH motif. The CC domains mediate protein–protein interaction and are typical of proteins functionally active in the cell as dimers or multimers. Also the LisH motif has been demonstrated to act as a dimerization motif (Kim *et al.*, 2004; Mateja *et al.*, 2006). To test whether OFD1 could self-associate, we performed co-IP experiments taking advantage of the different molecular weights of the MycGFP-tagged OFD1 compared with the Flag-tagged OFD1. The lysates from Cos-7 cells cotransfected with MycGFP-OFD1 and Flag-OFD1 were immunoprecipitated with an anti-Flag antibody, and the samples were analyzed by

Western blot with an anti-Myc (Figure 2A, left panel). The same lysates were immunoprecipitated with an anti-Myc, and the samples were immunoblotted with an anti-Flag (Figure 2A, right panel). The results showed that the anti-Flag antibody was able to immunoprecipitate MycGFP-OFD1 as well as the anti-Myc immunoprecipitated Flag-OFD1, thus further validating the interaction between the two differently tagged OFD1 forms. As a proof of specificity, the same IPs were performed using extracts from Cos7 cells transfected only with MycGFP-OFD1 or Flag-OFD1, respectively (Figure 2A).

To define the region responsible for homotypic interactions and the role of the LisH motif and the CC domains in mediating the OFD1 ability to self-associate, we used the interaction mating approach. For these experiments, we fused to both the LexA DNA binding domain (bait) and the B42 activation domain (prey) fragments encoding three different portions of the protein, namely OFD1a (containing only the LisH motif), OFD1b (comprising four predicted CC) and OFD1c (containing a single CC; Figure 2B). For the assay, only OFD1a and OFD1b were used, whereas the OFD1c fragment was excluded because of its ability to autoactivate transcription of reporter genes. The two yeast strains expressing, respectively, the bait and the prey proteins were mated, and the results showed that OFD1 is able to self-associate through its central region (OFD1b) where four CC domains concentrate. The OFD1a fragment did not interact with any of the tested constructs, suggesting that the LisH motif is not involved in OFD1 homotypic interactions (data not shown).

To confirm our findings, we performed GST pulldown experiments. For these experiments, we cloned OFD1a, b, and c, as well as an additional fragment comprising the LisH motif and three CC domains (OFD1d; Figure 2B), to be expressed as a GST fusion proteins. The rationale for generating this additional construct was to maintain the contigu-

Figure 2. OFD1 homotypic interactions. (A) Co-IP experiments. The lysates from Cos-7 cells cotransfected with vectors expressing Flag-OFD1 (120 kDa) and MycGFP-OFD1 (175 kDa) were immunoprecipitated with an anti-Flag antibody (left panel, lane 1) and as control, the lysates from cells transfected only with MycGFP-OFD1 were used (lane 2). The total cell lysates used as input are shown (TCL, lanes 1 and 2). The same experiment was repeated in the opposite direction, using an anti-Myc antibody for IPs and, as a control, the lysates from cells transfected with Flag-OFD1 (right panel, lanes 1 and 2, respectively). Also in this case total cell lysates used as input are shown (TCL, lanes 1 and 2). (B) OFD1 fragments used for interaction mating and GST pull-down experiments are represented with the corresponding domains LisH (dark gray) and CC (light gray). (C) GST pull-down assay in which the lysates from Cos-7 cells transfected with MycGFP-OFD1 was used as input and tested with the OFD1 fragments expressed as GST fused proteins (GST-OFD1a–d) and, as a control, with GST alone (GST). In the immunoblot, the MycGFP-tagged OFD1 corresponds to a band of about 175 kDa. IP, immunoprecipitated samples; IB, immunoblot. TCL+ and TCL– indicate the total cell lysates from cotransfected and not transfected Cos-7 cells, respectively.

ity of the LisH with the CC domains, in order to preserve eventually occurring intermolecular interactions that could generate a protein–protein interaction motif. In these experiments, Cos-7 cells were transfected with MycGFP-OFD1, and the cell lysate was tested with the four OFD1 fragments, after incubation with an equal amount of each GST fused protein or GST alone. As shown in Figure 2C, MycGFP-OFD1 was able to interact with GST-OFD1b but not with GST-OFD1a or GST-OFD1c, thus confirming that the fourth CC of the central region mediate the self-association, whereas the LisH motif and the OFD1 C-terminal region are not involved. It is worth noting that MycGFP-OFD1 did not interact with OFD1d, which lacks the fourth CC of the central region, thus showing that the presence of this CC domain is necessary for OFD1 homotypic interactions. This result also suggests that the LisH motif is not involved in OFD1 homotypic interactions even when associated with another dimerization motif.

The 1757delG mutation described in OFD type 1 patients (Ferrante *et al.*, 2001) results in a truncated form of the protein that resembles the OFD1d fragment, lacking the fourth CC domain and the C-terminal region (Figure 3A). This mutation was reproduced by site-directed mutagenesis and cloned to be expressed as bait or prey protein, in order to be tested with the OFD1b wild type in interaction mating assays. As expected, the 1757delG lost the ability to interact with OFD1b wild type. To further investigate this point, we expressed the 1757delG mutant as a MycGFP-tagged protein and tested its ability to interact with OFD1b fragment in GST pull-down experiments. The results show that the 1757delG

Figure 3. Study of the 1757delG OFD1 mutant form. (A) The position of the 1757delG frameshift mutation is depicted with respect to the LisH (dark gray) and the CC domains (light gray). (B) GST pull-down experiments. The lysates from Cos-7 cells overexpressing the MycGFP-tagged 1757delG mutant was used as input and tested with the OFD1b fragment expressed as GST fused protein and with GST alone. As control, lysates from cells overexpressing MycGFP-tagged OFD1 wild type were used. The band of about 175 kDa corresponds to the MycGFP-tagged OFD1 wild type, whereas the MycGFP-tagged 1757delG form (asterisk) is detectable as a band of about 100 kDa.

mutant is expressed at low levels in the cell, suggesting that the lack of ability of 1757delG to form homotypic interactions, which was demonstrated also by mating assay (data not shown), could be due to the amount of this mutant form available for molecular interactions (Figure 3B).

OFD1 Interacts with RuvB1, a Protein Belonging to the AAA⁺-Family of ATPases

To identify potential partners of OFD1, we used the yeast two-hybrid system to screen a HeLa cDNA library with the full-length OFD1 as bait. The experimental system used is the same we used for interaction mating experiments. This screening led to the isolation of 25 clones positive for the activation of the reporter genes. These clones corresponded to a partial cDNA encoding for RuvB1 (Gene Bank NM_003707), the evolutionarily highly conserved human protein that shows sequence homology to RuvB (a DNA-dependent ATPase and helicase in *Escherichia coli*) and that seems to mediate essential cellular functions (Shen *et al.*, 2000; Ikura *et al.*, 2000). To ensure that this interaction was specific, the full-length RuvB1 was expressed in yeasts as prey protein and tested with the original OFD1 full-length bait in interaction-mating experiments. These experiments confirmed the interaction (data not shown).

To determine whether the interaction of OFD1 with RuvB1 also occurs *in vivo*, we performed co-IP experiments in mammalian cells. We first investigated the interaction between the endogenous OFD1 and the overexpressed RuvB1. When lysates from Cos-7 cells transfected with a vector expressing HA-RuvB1 were immunoprecipitated with an anti-HA mAb, the immunocomplexes were found to contain endogenous OFD1, as shown in the immunoblot with the anti-OFD1 antibody (Figure 4A, left panels). Note that the anti-OFD1 antibody detects a doublet band probably due to the presence of alternative spliced forms of the protein, as already shown on protein extracts from total mouse embryo (Ferrante *et al.*, 2006), as well as in experiments performed using a different anti-OFD1 antibody raised against the C-terminal portion of the protein (Romio *et al.*, 2003).

Figure 4. OFD1 interaction with RuvB1. (A) Left panels, the lysates from Cos-7 cells transfected with a vector expressing HA-RuvB1 (top) or, as control, with the empty vector (bottom) were immunoprecipitated with a monoclonal anti-HA antibody, and the samples were immunoblotted with the polyclonal anti-OFD1. The experiment was repeated in the opposite direction (right panels) by using the anti-OFD1 C-ter antibody to immunoprecipitate extracts from cells transfected with a vector expressing MycGFP-RuvB1 (top) or with the empty vector (bottom). In B the lysates from MDCK cells were immunoprecipitated with the anti-OFD1 C-ter, the OFD1-associated proteins were eluted after competition with the antigenic peptide, and the samples were immunoblotted with the same anti-OFD1 antibody (left panel). The samples were then analyzed with anti-RuvB1 (right panel). As a control, the OFD1 antibody was incubated with the antigenic peptide prior IP (Ip control, both panels). In C, GST pull-down experiments. Overexpressed MycGFP-OFD1 was used as input and tested with GST-RuvB1 and GST alone. In (D) the different OFD1 fragments were expressed as GST fused proteins, and the lysates from Cos-7 cells overexpressing MycGFP-RuvB1 were used as input. IB, immunoblot; IP, immunoprecipitated samples; TCL, total cell lysate.

To verify that the OFD1 IP with the anti-HA antibody was specific, the same experiment was performed using lysates from cells transfected with the empty vector and in this experiment the anti-HA antibody failed to precipitate the endogenous OFD1 (Figure 4A, left panels). To perform the experiment in the opposite direction, we had to face the problem of distinguishing the band corresponding to HA-RuvB1 (about 50 kDa) from the one corresponding to the immunoglobulins (IgG) heavy chain, which display a similar molecular weight. We thus decided to test the interaction between the endogenous OFD1 and MycGFP-RuvB1, which has a molecular weight of about 80 kDa. Extracts from cells transfected with MycGFP-RuvB1-expressing vector or with the empty vector were immunoprecipitated with the anti-OFD1 C-ter, and the samples were immunoblotted with anti-Myc. The results from this experiment confirmed the interaction between the endogenously expressed OFD1 and MycGFP-RuvB1 (Figure 4A, right panels). We then set up the experiments to demonstrate that the endogenously expressed RuvB1 coimmunoprecipitates with the endogenous OFD1. For these experiments we first immunoprecipitated the lysates from MDCK cells with the anti-OFD1 C-ter antibody and, as a control, with the antibody preincubated with the immunogenic peptide. The immunoblot with the anti-OFD1 antibody showed that the preincubated anti-OFD1 was not able to immunoprecipitate the OFD1 protein (Figure 4B, left panel). To eliminate proteins corresponding to the IgG heavy chain, which have a molecular weight similar to that of the endogenous RuvB1, we eluted OFD1 immunocomplexes after competition with the OFD1 antigenic peptide, and we did not detect bands corresponding to the IgG heavy chain in the immunoprecipitates (Figure 4B, left panel), thus showing that IgG removal was successfully achieved. Finally, the same immunoprecipitates were analyzed with a

polyclonal anti-RuvB1, and the results showed the interaction between both the endogenous expressed proteins. Also in this case, no IgG were detected in the control sample (Figure 4B, right panel).

To investigate if OFD1 and RuvB1 could directly interact and to identify the regions of the OFD1 protein involved in this interaction, we performed GST pull-down experiments. In a first set of experiments, we cloned the full-length *RuvB1* cDNA to be expressed as a GST fused protein, and the lysate from Cos-7 cells expressing the full-length MycGFP-tagged OFD1 was used as input in the assay. This experiment showed that the overexpressed OFD1 protein is able to interact with GST-RuvB1 (Figure 4C). In a second set of experiments, we expressed the OFD1a, b, c, and d fragments as GST-fused proteins and used MycGFP-RuvB1-transfected Cos-7 cell lysates as input. The cellular lysates were incubated with an equal amount of each GST fused protein or, as a control, with GST alone. MycGFP-RuvB1 was found to bind GST-OFD1b, c, and d, thus suggesting that, in addition to the central region, the OFD1 C-terminal region might be relevant for the interaction with RuvB1 (Figure 4D). It is interesting to note that RuvB1 interacts with GST-OFD1d, which lacks the fourth CC of the central region and the C-terminal region, suggesting that the lack of the fourth CC domain exerts different effects in OFD1 homo- and heterotypic interactions and that the C-terminal region is involved but not essential for OFD1-RuvB1 interaction.

OFD1 Interacts with Subunits of the TIP60 Chromatin-remodeling Complex

RuvB1 has been isolated in multiprotein complexes involved in chromatin remodeling (Shen *et al.*, 2000), among which is the TIP60 complex (Ikura *et al.*, 2000). TIP60 is the human HAT multisubunit complex, homologous to the well-known NuA4 complex characterized in yeast (Doyon *et al.*, 2004). Starting from the OFD1 interaction with RuvB1 and in order to find a functional link to the presence of OFD1 in the nucleus, we investigated whether OFD1 is also part of the TIP60 nuclear complex. Protein extracts from MDCK cells were subjected to IP with the anti-OFD1 polyclonal antibody. Bound proteins were eluted with the OFD1 peptide, and the samples were analyzed by Western blot with antibodies against four additional subunits of the TIP60 complex from the 14 known to date, namely RuvB2, DMAP1, TRRAP, and TIP60. Our results showed that OFD1 is able to coimmunoprecipitate each of these proteins (Figure 5A). The same experiment performed with the anti-OFD1 preincubated with the immunogenic peptide failed to recover the TIP60 subunits, as already demonstrated for OFD1. In Figure 5B, two of these control IPs are reported. Moreover, to further strengthen the observation that OFD1 interacts with subunits of the TIP60 complex, we demonstrated that IP with an antibody directed against the TIP60 subunit was able to recover the OFD1 endogenous protein (Figure 5C). All these data strongly suggest that the OFD1 protein is part of TIP60 chromatin remodeling complex, further validate the interaction between OFD1 and RuvB1 and also indicate a possible new function of the OFD1 protein.

DISCUSSION

The aim of this work was to undertake a preliminary functional characterization of the OFD1 protein.

Figure 5. OFD1 coimmunoprecipitates with subunits of the human TIP60 complex. (A) Lysates from MDCK cells were immunoprecipitated with the anti-OFD1 Cter antibody, eluted with OFD1 peptide, and analyzed by Western blot with antibodies recognizing four subunits of the TIP60 complex. OFD1 coimmunoprecipitates with TRRAP, TIP60, DMAP, and RuvBI2. (B) Two examples of the control experiments in which the same lysates were immunoprecipitated with the anti-OFD1 antibody preincubated with the peptide (Ip control). (C) Immunoprecipitation with an anti-TIP60 antibody recovered the endogenous OFD1 protein. IP, immunoprecipitated samples; TCL, total cell lysate.

OFD1 Has Both a Ciliary and Nuclear Localization

OFD1 has been localized to the centrosome in human embryonic renal mesenchymal lines (Romio *et al.*, 2003) and more recently OFD1 has been found to be located at the basal bodies and at the axoneme of primary cilia in proximal tubule cell lines (Romio *et al.*, 2004). We have also showed that in ciliated MDCK cells, OFD1 localizes to the basal body of the primary cilium although we did not detect signal in the axoneme with our anti-OFD1 antibody.

Cilia are highly conserved organelles with diverse motility and sensory functions, which have been recently shown to display crucial roles in cell signaling pathways and in maintaining cellular homeostasis. As such, defects in cilia formation or function results in impairment of several developmental processes and the physiology of multiple organ systems. To date ciliary dysfunction has been associated to a wide spectrum of developmental and adult phenotypes, with mutations in ciliary proteins now associated with nephronophthisis (NPHP), polycystic kidney disease (PKD), reversal or randomization in body symmetry, Bardet-Biedl (BBS), Senior-Loken, Joubert, Meckel-Gruber and Alstrom syndromes (Badano *et al.*, 2005, 2006; Beales, 2005; Hildebrandt and Otto, 2005; Bisgrove and Yost, 2006 for a review). NPHP, PKD, Joubert, Senior-Loken, and BBS display cystic kidney, which is present in ~15% of OFD1 patients (Gorlin, 1990), whereas the limb abnormalities, which are a distinctive sign of OFD type I syndrome, are also present in BBS, Meckel-Gruber, and Joubert patients. The localization of the OFD1 protein to the primary cilium correlates well with data from the literature, and we have recently demonstrated, that, indeed, Ofd1 is required for primary cilia formation and left-right axis specification (Ferrante *et al.*, 2006). These data definitively place OFD1 syndrome in the group of genetic disorders associated to ciliary dysfunction.

Moreover, here we report, for the first time, that OFD1 is also located to the nucleus. Interestingly, also inversin, which is responsible for the infantile form of NPHP, and polycystin-1, which are involved in autosomal dominant PKD, is localized both to the primary cilia and to the nucleus (Nurnberger *et al.*, 2002; Yoder *et al.*, 2002; Otto *et al.*, 2003; Guay-Woodford, 2004).

OFD1 Displays Homotypic and Heterotypic Interactions

Immunofluorescence and direct fluorescence experiments demonstrate that when overexpressed OFD1, in addition to its centrosomal localization, shows a punctate pattern concentrating in speckles of variable size uniformly distributed in the cytoplasm (data not shown). This pattern is typical of proteins able to form aggregates, because CC-rich proteins do (Chao *et al.*, 1998). Co-IP experiments demonstrate that OFD1 is able to self-associate. Interaction mating and GST pulldown experiments indicate that the LisH motif does not have a role in the homotypic interactions, which are rather mediated by the four CC domains, located within the central region. In particular, the study of the OFD1d fragment suggests that the fourth CC domain is critical for these interactions.

Yeast two-hybrid screening resulted in the identification of RuvBI1 as molecular partner of the OFD1 protein, and their interaction was confirmed in mammalian cells. The study of the OFD1 fragments indicates that the CC domains of the central region, already described to be responsible for homotypic interaction, mediate also the OFD1-RuvBI1 interaction and that the OFD1 C-terminal region is also involved in this interaction. In particular, the study of the OFD1d fragment indicates that the C-terminal region and the fourth CC domain are important, although not essential, for the hetero-interaction. Because of the structural identity between the OFD1d fragment and the 1757delG OFD1 mutant form, we could infer that this mutant form retains the ability to interact with RuvBI1, whereas, as demonstrated, it lacks the ability to associate with OFD1 wild type. It is tempting to speculate that an impairment of the OFD1 homo-interaction properties could represent the pathogenetic mechanism in this mutation, although additional studies will be needed to confirm this hypothesis.

The presence of several CC domains in the OFD1 protein correlates well with its ability to homo- and hetero-interact, and it is conceivable that OFD1 exerts its biological function in the cell as part of multiprotein complexes. In these complexes, we hypothesize that OFD1 homo and heterotypic interactions take place according to a stoichiometric balance, which must be preserved for the correct functioning of the complexes themselves.

OFD1 Interacts with RuvBI1 and Other Subunits of the TIP60 HAT Multisubunit Complex

RuvBI1 is an highly conserved human protein belonging to the AAA⁺-family of ATPases and to which several biological functions have been assigned. RuvBI1 shows structural homology to bacterial RuvB ATPase, but its ATPase activity has been widely questioned, and at the present its biochemical role remains undefined (Matias *et al.*, 2006). Recently, *RuvBI1* has been identified as a gene causing polycystic kidneys during an insertional mutagenesis screen in zebrafish (Sun *et al.*, 2004). Further data connecting *RuvBI1* to the primary cilium come from a genome-wide analysis of the RNA transcriptional profile observed in *Chlamydomonas reinhardtii* during the regeneration of flagella, which are virtually identical to human cilia. This analysis revealed that RNA transcription of the *RuvBI1* homolog in *Chlamydomonas*

is strongly induced during flagellar regeneration. On the basis of these findings the authors suggest a functional role of this transcript in the process of cilia formation and function and they also propose that RuvBl1 may act in the nucleus either to direct the transcriptional program of flagellar assembly or to mediate gene regulation in response to sensory cilia functions (Stolc *et al.*, 2005). In addition, the murine homolog of RuvBl1 has been recently identified as one of the components of the photoreceptor sensory cilium during a proteomic analysis (Liu *et al.*, 2007).

RuvBl1 has been isolated in complexes with proteins involved in chromatin remodeling (Ikura *et al.*, 2000; Shen *et al.*, 2000), and it shows a preponderant nuclear localization (Holzmann *et al.*, 1998). Here we report that OFD1 also localizes to the nucleus. Immunofluorescence experiments showed that the nuclear signals of the endogenous OFD1 and RuvBl1 partially overlap, but the signal in the nucleoplasm of both proteins is so diffuse that it is difficult to define their colocalization (data not shown). However, the finding that OFD1, together with RuvBl1, coimmunoprecipitates with proteins associated in the TIP60 chromatin remodeling complex prompt us to hypothesize that OFD1 and RuvBl1 could interact at the nuclear level.

We demonstrated that OFD1 self-associates and interacts with RuvBl1, RuvBl2, DMAP, TRRAP, and TIP60, all subunits of the TIP60 complex, the human HAT multisubunit complex homologous to the yeast NuA4 complex. We hypothesize that also OFD1 could be part of this complex, but further experiments are required to define its role within the complex. As an HAT complex, TIP60 controls multiple key nuclear functions in eukaryotic cells. Many subunits of this complex have been linked to the global and targeted histone H4 acetylation *in vivo* and to the regulation of transcription and cell cycle progression, as well as to the process of DNA repair and apoptosis (reviewed in Doyon and Cote, 2004). OFD1, as part of this complex, could act in the nucleus to regulate the transcriptional program that directs the development of tissues and organs affected in OFD type 1 syndrome. The structural or functional impairments of this HAT complex and the consequent genetic deregulation could explain the development of cystic kidney as well as of the other clinical signs associated with this disorder. Interestingly, a novel LisH-like motif has been shown to be involved in histone H4 transcriptional activity (Wei *et al.*, 2003). The LisH is the most characteristic structural motif recognized in the OFD1 protein. Here we show that it does not mediate OFD1 homo- and heterotypic interactions, and we speculate that this motif might have a role in the still undetermined nuclear function of the OFD1 protein.

Several studies have shown that polycystin-1 and -2, nephrocystin, and inversin, responsible for different genetic forms of cystic kidneys, are primary cilium proteins that activate intracellular signaling cascades, and these intracellular signals culminate in gene expression, finally regulating cell proliferation and differentiation (Wilson, 2004). Here we propose that OFD1, in addition to its ciliary function, could exert a direct role in the nuclear compartment as it has been hypothesize for other ciliary proteins (Nurnberger *et al.*, 2002; Guay-Woodford, 2004). A very interesting query that remains to be solved is whether the ciliary and the nuclear functions of the OFD1 protein are related or act on independent pathways. Further investigation will be needed to define the role of the OFD1 protein both in the nucleus and in the cilia and to address the numerous questions that emerged from the present work.

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