Ofd1 is required in limb bud patterning and endochondral bone development

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A B S T R A C T

Oral–facial–digital type I (OFD1; MIM 311200) syndrome belongs to the heterogeneous group of developmental disorders known as oral–facial–digital syndromes (OFDS) (Gurrieri et al., 2007; Macca and Franco, 2009). OFD1 has an estimated incidence of 1:50,000 live births (Wahrman et al., 1966) and is transmitted as an X-linked male lethal developmental disorder. It is ascribed to ciliary dysfunction and characterized by malformation of the face, oral cavity, and digits. Conditional inactivation using different Cre lines allowed us to study the role of the Ofl1 transcript in limb development. Immunofluorescence and ultrastructural studies showed that Ofl1 is necessary for correct ciliogenesis in the limb bud but not for cilia outgrowth, in contrast to what was previously shown for the embryonic node. Mutants with mesenchymal Ofl1 inactivation display severe polydactyly with loss of antero-posterior (A/P) digit patterning and shortened long bones. Loss of digit identity was found to be associated with a progressive loss of Shh signaling and an impaired processing of Gli3, whereas defects in limb outgrowth were due to defective Ihh signaling and to mineralization defects during endochondral bone formation. Our data demonstrate that Ofl1 plays a role in regulating digit number and identity during limb and skeletal patterning increasing insight on the functional role of primary cilia during development.

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Introduction

Oral–facial–digital type I (OFD1; MIM 311200) syndrome belongs to the heterogeneous group of developmental disorders known as oral–facial–digital syndromes (OFDS) (Gurrieri et al., 2007; Macca and Franco, 2009). OFD1 has been shown to be localized to the basal body at the origin of centrosome-associated protein (Romio et al., 2003). More recently, OFD1 has been shown to be localized to the basal body at the origin of primary cilia (Giorgio et al., 2007; Romio et al., 2004).

Primary cilia are complex and evolutionarily conserved eukaryotic organelles, present on nearly all mammalian cells. They extend from, and are continuous with, the cell membrane. Ciliary proteins are synthesized in the cell body and transported to the tip of the ciliary axoneme by intraflagellar transport (IFT), a highly regulated anterograde and retrograde translocation of polypeptide complexes along the length of the axoneme (Badano et al., 2005; Ferrante et al., 2001). IFT encodes for a 1011-amino-acid protein characterized by the presence of five predicted CC domains that occupy almost the entire length of the molecule and a Lis1 homology motif (LisH), shared with over 100 eukaryotic intracellular proteins, at the N-terminal region (Enes and Ponting, 2001; Gerlitz et al., 2005). Previous studies have shown that OFD1 co-localizes with γ-tubulin, suggesting that it is a centrosome-associated protein (Romio et al., 2003). More recently, OFD1 has been shown to be localized to the basal body at the origin of primary cilia (Giorgio et al., 2007; Romio et al., 2004).

Emerging data suggest that cilia act as cellular antennae with diverse motility and sensory functions, which detect a wide variety of signals. Recent studies have shown that cilia have crucial roles in different signal transduction pathways such as Hedgehog (Hh), canonical and non-canonical (planar cell polarity or PCP) Wnt, and platelet-derived growth factor (PDGF) pathways. Primary cilia are complex and evolutionarily conserved eukaryotic organelles, present on nearly all mammalian cells. They extend from, and are continuous with, the cell membrane. Ciliary proteins are synthesized in the cell body and transported to the tip of the ciliary axoneme by intraflagellar transport (IFT), a highly regulated anterograde and retrograde translocation of polypeptide complexes along the length of the axoneme (Badano et al., 2005; Gerdes and Katsanis, 2005). Emerging data suggest that cilia act as cellular antennae with diverse motility and sensory functions, which detect a wide variety of signals. Recent studies have shown that cilia have crucial roles in different signal transduction pathways such as Hedgehog (Hh), canonical and non-canonical (planar cell polarity or PCP) Wnt, and platelet-derived growth factor (PDGF) pathways.
factor (PDGF) signaling (Bisgrove and Yost, 2006; Eegenschieler and Anderson, 2007; Gerdes et al., 2009). Cilia were shown to be present on both ectodermal and mesenchymal cells in the developing limb and on the chondrocytes of long bones (Haycraft et al., 2007). Accordingly, defects in cilia formation or function have profound effects on the development of body pattern and on the physiology of multiple organ systems and underlie a wide range of human disorders, all of which present polydactyly as common feature (for a review see, Badano et al., 2006; Bisgrove and Yost, 2006; Gerdes et al., 2009).

Ofd1-knockout mice have been generated. Heterozygous females (Ofd1^{+/−−}/−) reproduced the main features of the human disease, albeit with increased severity, probably due to differences in X-inactivation patterns observed between the two species (Ferrante et al., 2003; Morleo and Franco, 2008). Female mutants died at birth, displaying craniofacial and skeletal abnormalities including polydactyly at both limbs. Instead, the Ofd1^{−/−} male mutants died early during gestation, displaying neural tube closure defects and defective formation of nodal cilia resulting in abnormal specification of the left–right body asymmetry (Ferrante et al., 2006).

Conditional loss of IFT transcripts in the limb mesenchyme resulted in abnormal patterning of the autopod during early stages of limb morphogenesis and in appendicular long bone defects at later stages of limb development (Haycraft et al., 2007). Very recently, the role of IFT and primary cilia has been postulated in the development of the postnatal growth plate (Song et al., 2007). Recent studies have demonstrated the role of basal body proteins in limb development. Fim, a basal body protein involved in limb development and left–right axis specification, is not essential for cilia assembly but plays a role in Shh signaling (Vierkotten et al., 2007). EVC is a basal body protein mutated in Ellis-van Creveld syndrome, a human chondroectodermal dysplasia characterized by limb and skeletal abnormalities, which are well reproduced in the Evc^{−/−} mouse model. Characterization of these mice allowed to demonstrate that defective transduction of Indian hh (Ihh) signaling is involved in the skeletal phenotype associated to Evc mutation (Ruiz-Perez et al., 2007).

The embryonic male and perinatal female lethality observed in Ofd1 mutants has so far prevented experiments aimed at elucidating the role of the Ofd1 transcript in limb development. We therefore sought to generate conditional models with Ofd1 limb-specific inactivation. In this study, we show that conditional inactivation of Ofd1 in the apical ectodermal ridge (AER) did not result in limb abnormalities, whereas conditional inactivation in limb mesoderm resulted in mutant animals displaying polydactyly and shortened long bones, with progressive loss of Shh signal transduction accompanied by impairment of Gi3 processing and malformed cilia. The long bones displayed defects in chondrocytes organization, reduction in mineralization process, and defective Ihh signaling.

Our data strongly indicate that Ofd1 has an important role in regulating digit number and identity during limb and skeletal patterning and contribute insight into the functional role of primary cilia during development.

Materials and methods

Mouse strains and PCR genotyping

The generation of Ofd1^{fl/fl}, Msx2Cre, and of Prx1Cre mice has been previously described (Ferrante et al., 2006; Logan et al., 2002; Sun et al., 2002). We crossed an Ofd1^{fl/fl} female with a Msx2Cre male to obtain Ofd1^{fl/fl−−}Msx2Cre females and Ofd1^{fl/fl−−}Msx2Cre males and crossed an Ofd1^{fl/fl} female with a Prx1Cre male to obtain Ofd1^{fl/fl−−}Prx1Cre females and Ofd1^{fl/fl−−}Prx1Cre males. For genotyping, DNA was extracted from extra-embryonic membranes and tails of embryo and adult mice. Animals were genotyped as previously described (Ferrante et al., 2006). In all timed pregnancies, plug date was defined as E0.5. All animal experimentation was done following the regulation of the Animal Care and Use Committee of Cardarelli Hospital (Naples, Italy) and authorized by the Italian Ministry of Health.

RNA extraction, cDNA synthesis, and real-time RT-PCR data analysis

Total RNA was made using TRIzol (Invitrogen), and cDNA was synthesized from 2 μg of DNase1-treated total RNA using SuperScript III (Invitrogen) and oligo-DT_{12-18} (Promega) according to the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) was carried out using the Applied Biosystems SYBR-green dye system and 7500 Real Time Cycler in 96-well plates. Cycling parameters were as follows: 50 °C for 2 min, 95 °C for 10 min, then 40 cycles of 15-s denaturation at 95 °C and 1-min at 60 °C annealing temperature. Expression levels were normalized against mouse hypoxanthine phosphoribosyltransferase (HPRT) as determined from the ratio of ΔCT values. Primers used were HPRT-F 5′-GGATGATGCCAACAAAATGG-3′; HPRT-R 5′-GAATAAGCTGCGCTTATCCTCA-3′; GI1-F 5′-GCTTGGGATAGAGCACGCTTGC-3′; GI1-R 5′-GCTGATCCGCTAAGGTTCTC-3′; Ofd1-F 5′-CAATCTCTTGATATTTGGAGG-3′; Ofd1-R 5′-GTGTTAGAGGGTTAGAACACATG-3′; Ptc1-F 5′-GGAAATGACAACATGG-3′; Ptc1-R 5′-TCCTCCTCTGCTGCTCCCT-3′; Shh-F 5′-GGAAATCTACCCCAATACA-3′; Shh-R 5′-TCTCATCAAGATGACCAAG-3′. Mean of relative expression ± SD was determined from biological replicates (n = 2–4 samples of two forelimbs each). Statistical significance was determined using a Student’s t-test (*p < 0.05). All amplicons were gel-purified and sequenced to ensure correct product amplification.

Whole-mount in situ hybridization analysis

Whole-mount in situ hybridization was carried out as described (Ferrante et al., 2006) using digoxigenin-labeled antisense RNA probes. Each experiment was performed on at least three independent embryos. All pictures were acquired at the same magnification.

Immunofluorescence

For analysis of cilia in vivo, limbs were dissected from E10.5 and E11.5 wt and mutants, fixed in 4% paraformaldehyde (PFA), embedded in OCT, and snap frozen. Sections of 20 μm were cut, incubated for 30 min with 0.1% BSA, 10% goat serum in Tris-buffered saline (TBS) and then with 1 mg/ml hyaluronidase (Sigma Aldrich, St Louis, MO) at 37 °C for 45 min before incubation with anti-acetylated tubulin antibody (1:2000 Sigma) overnight at 4 °C. Sections were then washed with TBS and incubated with secondary anti-mouse IgG conjugated to Cy3 (Jackson ImmunoResearch). Stained sections were mounted with Vectashield (Vector Laboratories). Microscopy was performed with a Zeiss Axiosplan 2 microscope and with a Leica TCS SP2 AOBS scanning confocal microscope with a 63× Neofluor Pan-Apo 1.3 nm oil lens.

Morphometric analysis

Morphometric analysis (Ascenzi et al., 2007) was conducted on stacks of confocal images obtained from two animals of each E10.5 mutant, E11.5 mutant, and controls. Briefly, each stack of images was imported into vector graphic editor XaraX1 software (XaraX Co, London) and a three-dimensional (3D) reference system was assigned to each stack. On each image, a segment was drawn to overlap each stack of confocal images obtained from two animals of each E10.5 mutant, E11.5 mutant, and controls. Brie

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length of each segment in real microns by means of a calibration formulated in terms of confocal settings. The length of each cilium was assessed by using the distance formula in 3D from the point of the stack’s first segment closer to the cell to the end of the last segment further from the cell. The statistical Student’s t-test with significance set at 0.05 was employed to normalized data to establish differences in ciliary length between mutants and controls. Diagrams were prepared to display the data.

**Transmission electron microscopy (TEM)**

Forelimbs were cut longitudinally in two pieces in 0.1 M pH 7.2 phosphate buffer (PB) to which 1.8% sucrose was added. Samples were fixed for 24 h at 4 °C in 2.5% glutaraldehyde in PB and then rinsed in buffer and post-fixed in 1% osmium tetroxide in PB for 1 h at 4 °C. After dehydration in a graded series of ethanol, samples were embedded in an Epon-Araldite mixture. Ultrathin sections, obtained with a Reichert Ultracut II E and an LKB Ultratome III, were stained (1% uranyl acetate following 1% lead citrate) and observed with a Philips CM 10 transmission electron microscope operating at 80 kV.

**Western blotting**

For Western blotting analysis of Gli3, whole-cell lysates were prepared from dissected forelimbs and hindlimbs of E11.5 wt, Ofd1fl+/y [Prx1Cre, Ofd1fl+/y]Prx1Cre using RIPA buffer [10 mM Na-phosphate pH 7.2, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Na-deoxycholate, 0.1% SDS, protease inhibitors cocktail (Roche)]. Limb buds pooled from wt and mutant embryos were disrupted using a mini-pestle (Bio-Optica) prior to protein extraction. Equal amounts of protein were loaded onto 7% SDS-PAGE gels, and Western blotting was performed as described (Wang et al., 2000). The polyclonal affinity-purified Gli3 antibody was a gift from S. Mackem of Laboratory of Pathology and Cancer & Developmental Biology, in Bethesda. Membranes were then stripped and probed using an antibody against β-tubulin (Sigma #T4026) as loading control. Densitometry was used to compare protein levels between the full-length activator and truncated repressor forms of Gli3 (ImageJ 1.37v software available at http://nih图像). The Ofd1 antibody was used as previously described (Ferrante et al., 2006; Logan et al., 2002; Sun et al., 2002).

**Histology, BrdU, and TUNEL analyses**

Limbs were dissected from E16.5 wt and conditional mutant embryos, fixed in 4% PFA overnight at 4 °C and embedded in paraffin for sectioning using standard procedures. Sections of 18 μm were stained with hematoxylin and eosin (H&E) and with Safranin O according to standard procedures. For detection of mineralization, fixed limbs from E16.5 wt and conditional mutants were isolated, washed with several changes in PBS, and infiltrated with 30% sucrose in PBS overnight at 4 °C. Sucrose-equilibrated samples were embedded in OCT (Kaltek) and 20-μm sections were sectioned on a Leica CM3050S cryostat (Leica Microsystems Gmth, Wetzlar, Germany). Sections were stained with 1% silver nitrate (Von kossa method) and counterstained with Methyl Green (Sigma). H&E and Von Kossa stained sections were visualized on a Zeiss Imager A1 microscope and images were captured with Axiocam HRc digital camera. E16.5 pregnant females were sacrificed 2 h after receiving an intraperitoneal injection of BrdU cell proliferation labeling reagent (GE Healthcare). Proliferating cells were detected on 15-μm cryosections by immunohistochemistry (BrdU Staining Kit, Zymed), BrdU-positive and BrdU-negative nuclei were counted in columnar chondrocytes of Ofd1fl+/yPrx1Cre and wt mice (seven sections for both). The percentage BrdU-positive cells was calculated for each section and data compared by ANOVA. Detection of apoptosis was performed by a TUNEL assay using the in situ cell death detection kit (Roche) according to the manufacturer’s instructions. Nuclei were counterstained with DAPI and sections were examined by fluorescence microscopy.

**In situ hybridization analysis**

In situ hybridization was performed on 15 μm paraffin embedded sections as previously described (Hill et al., 2005). For Pthr and Ofd1, we used radioactive-labeled (35 S) antisense RNA probe following standard procedures. After stringency washes, dehydrated sections were coated with nuclear emulsion (K5, Ilford) and exposed for 2–4 weeks at 4 °C. The signal was developed in Kodak D-19 developer and sections counterstained with nuclear Fast Red. Bright-field images were obtained on a Nikon DS-5 L1 digital camera and dark fields were produced in Photoshop (Adobe).

**Results**

**Conditional inactivation of Ofd1 in limb mesenchyme results in polydactyly and shortened long bones**

Heterozygous mutant females (Ofd1fl+/−) displayed polydactyly and skeletal abnormalities and died at birth, while hemizygous male mutants (Ofd1fl/y) died at E11.5–E12.5 with severe developmental defects (Ferrante et al., 2006). To circumvent the problem of embryonic male and perinatal female lethality and in order to study the role of Ofd1 in limb development, we sought to generate a conditional line with Ofd1 limb-specific inactivation. To select the most appropriate line, we analyzed Ofd1 expression in mouse developing limb buds. RT-PCR and whole-mount in situ experiments indicated that Ofd1 is expressed from E10.5 in both limbs (data not shown), with a higher expression at E11.5. At this stage, Ofd1 expression was predominantly found in the mesoderm (Fig. S1A–B). By E12.5, Ofd1 expression begins to appear in the interdigital zone of the autopod (arrows in Fig.S1C). At E16.5 Ofd1 expression was detected in the perichondrium of the developing bones (Fig. S1E). These data suggest that Ofd1 could play a role in mesenchymal-derived structures during limb development. To directly investigate the function of Ofd1 in limb bud mesoderm, we crossed Ofd1fl/y female mice with Prx1Cre transgenic male mice that express the Cre recombinase in the limb mesenchyme from E9.5, when the expression of Prx1Cre is predominant in the forelimb mesenchyme. By E10.5, the expression is evident in both limbs (Logan et al., 2002). Mutants were viable and only displayed limb abnormalities. The expression of Ofd1 cDNA in the wild-type (wt) and mutant forelimbs was determined by real-time PCR at E10.5 and E11.5 to quantify the efficiency of the knockdown. Our analysis revealed the presence of 80% and 90% of the wt allele on RNA of forelimbs from Ofd1fl/flPrx1Cre and Ofd1fl/+Prx1Cre mutants, respectively, when compared to controls at E10.5 (Fig. S2A). At E11.5, we found the presence of 20% and 70% of the wt allele on RNA from forelimbs from Ofd1fl/flPrx1Cre and Ofd1fl/+Prx1Cre mutants, respectively, when compared to wt (Fig. S2B). To verify that the loss of the expression of Ofd1 cDNA in mutants correlates with the loss of Ofd1 protein, we isolated forelimbs from E11.5 mutant mice and compared the amount of Ofd1 protein to that found in forelimbs from wt mice, by Western blot analysis using an antibody for Ofd1 as previously described (Ferrante et al., 2006). In agreement with the real-time PCR experiments, the amount of Ofd1 protein was not reduced at E10.5 in the forelimbs of Ofd1fl/flPrx1Cre mutants (lane 3 in Fig.S2C), while at E11.5, the level of Ofd1 protein is dramatically reduced in forelimbs of Ofd1fl/flPrx1Cre mutants (lane 3 in Fig.S2D). The low level of Ofd1 protein observed in Ofd1fl/flPrx1Cre conditional mutants could be explained either due to a small number of limb bud mesenchymal cells where recombination has not occurred or due to the fact that recombination has just taken place and some residual levels of Ofd1 protein could be still present. The blot was simultaneously probed for β-tubulin to ensure similar loading of wt and mutant samples. Altogether
these data indicate that Ofd1 inactivation in limb mesenchyme is effective by E11.5. The limb abnormalities were more evident in the forelimbs than in the hindlimbs, possibly due to differences in Cr activity between limbs previously reported for this line (Logan et al., 2002). However, we cannot exclude a different role of Ofd1 in forelimbs and hindlimbs. Newborn Ofd1fl/flPrx1Cre females displayed proxial polydactyly of the first digit in each forelimb and normal hindlimbs (Fig. 1E–H). As expected, Ofd1fl/flPrx1Cre males were more severely affected than females. Malformations in the autopod included severe polydactyly with 7 to 9 unpatterned digits on each forelimb and a single extra digit on each hindlimb (Fig. 1I–L). In addition, Ofd1fl/flPrx1Cre mutants displayed shortened long bones along the proximodistal axis (Fig. 1I, L, K). All the sixteen newborn mutant mice (eight males and eight females) from different littersmates observed to date displayed the abnormal phenotype described above. Two out of eight male mutants also displayed syndactyly in the forelimbs (arrow in Fig. 1J). The skeletal abnormalities described above. Two out of eight male mutants also displayed syndactyly in the forelimbs (arrow in Fig. 1J). The skeletal abnormalities observed in Ofd1 type I patients although with a higher degree of severity. We observed mutants up to P17. After this stage, mutant animals, which were severely malformed, were sacrificed. Skeletal analysis with alizarin red at P17 showed that all the skeletal elements of the limbs were completely formed in mutants, although shorter when compared to wt (data not shown), suggesting that proximodistal limb patterning is independent of Ofd1 function in the mesenchyme. To address the role of Ofd1 in the apical ectodermal ridge (AER) and in the ventral ectoderm, we generated conditional mutants by crossing the Ofd1fl/female mice with the Mox2Cre transgenic male mice. Expression of Cre in this transgenic line is restricted to the AER and in the ventral ectoderm starting from E9.5–10 (Sun et al., 2002). Limbs of Ofd1fl/fMox2Cre mutants were indistinguishable from wt mice (data not shown). Altogether, our data indicate that Ofd1 does not play a role in AER and ventral ectoderm patterning and outgrowth, although we cannot exclude a role for Ofd1 at earlier stages or in the dorsal ectoderm.

**Fig. 1.** Skeletal phenotype in Ofd1fl/flPrx1Cre mutants at P0. Alizarin red and Alcian blue stainings identify the skeletal elements of the limbs. Stylopods and zeugopods from Ofd1fl/flPrx1Cre are quite normal (E, G). The autopod from Ofd1fl/flPrx1Cre is polydactylous with duplication of digit 1 (asterisks in E–F) in the forelimb. Ofd1fl/flPrx1Cre stylopod and zeugopod elements are dramatically affected in both limbs (I–L), displaying mineralization defects. Syndactalty (arrow in J) can be recognized in the forelimb with shortened and unpatterned digits while autopods in the hindlimb are polydactylous (asterisks in L). Scale bar: 100 μm. Abbreviations: fe, femur; fl, fibula; hu, humerus; ra, radius; ti, tibia; ul, ulna.

**Ofd1 inactivation in the limb mesenchyme is associated to cilia dysfunction**

Dysfunction of primary cilia has been associated with limb and skeletal abnormalities (Haycraft et al., 2005; Haycraft et al., 2007; Koyama et al., 2007; McGlashan et al., 2007). We thus performed an immunofluorescence analysis with the anti-acetylated tubulin antibody, which stains stabilized microtubules, including the cilary axoneme, on forelimb sections of Ofd1 conditional mutants and wt at E11.5. Cilia were observed in the mesenchyme of Ofd1fl/flPrx1Cre mutant forelimbs (Fig. 2B–C) although they appeared shorter than in the wt (Fig. 2A). A morphometric analysis, performed on the confocal images, quantified the significant different ciliary length (p < 0.01) between Ofd1fl/flPrx1Cre conditional mutant and wt. Similar results were obtained when primary cilia from the mesenchyme of Ofd1fl/fl null male mutants were analyzed at E10.5 (data not shown). We also analyzed the cilia in the AER and in the ventral ectoderm of Ofd1fl/fl and Ofd1fl/flPrx1Cre limb mutants. No significative differences (p = 26) in ciliary lengths were observed between Ofd1 mutant animals (both null and conditional) and control animals (Fig. S3 and data not shown). These results further support the specific role of Ofd1 in limb mesenchyme.

To further address the role of Ofd1 in cilia formation in the limb bud, a transmission electron microscopy (TEM) analysis was performed on conditional mutants and controls at E11.5. Cross-sections showed an almost compact tissue consisting of mesenchymal cells limited by narrowed intercellular space and form thin protusions. Some cells show their nuclei with the...
The typical appearance of mitotic division. A centrosome that consists of two centrioles with a variable reciprocal position is often visible in the cytoplasm. The centrioles show the conventional microtubular organization in a cartwheel array surrounded by amorphous dense material (Fig. S4A). Occasionally two couples of centrioles are observed in the same cytoplasm of cells at the initial stage of mitotic division. The primary cilia are not easy to find: this is the first example of a TEM study on primary cilia of limb bud during early stages of development, to the best of our knowledge. Most of the findings represent the initial steps of ciliogenesis characterized by the presence of the basal body, which is close to the cell surface and gives rise to the formation of a cytoplasmic bud filled with dense material. The complex is contained in a ciliary pocket (Fig. S4B). Growing cilia can be found at the end of the elongating process (Fig. 3A) and can reach up to 2.4 μm in length. The 9+0 axoneme with doublet microtubules lacking dynein arms is shown in a cross-section of the basal ciliary shaft (Fig. 3B). The analysis of a longitudinal section of the ciliary shaft of primary cilia from Ofd1fl/+|Prx1Cre female shows that some microtubular

Fig. 2. Cilia of Ofd1 mutants are shortened in limb mesenchyme. Immunofluorescence analysis with the acetylated α-tubulin antibody stains primary cilia on sections of forelimb mesenchyme at E11.5 in wt (A) and Ofd1fl|Prx1Cre mutants (B–C). (D) Morphometric analysis revealed that a significant statistical difference in primary cilia length of mesenchymal cells exists between Ofd1fl|Prx1Cre (lane 3) and wt (lane 1) (1.38 ± 0.28 μm vs. 2.82 ± 0.41 μm; p < 0.01) and between Ofd1fl|Prx1Cre (lane 2) and wt (1.99 ± 0.61 μm vs 2.82 ± 0.41 μm; p < 0.01). Scale bar 2 μm in panels A–C.

Fig. 3. Ultrastructural analysis of primary cilia from limb buds of E11.5 Ofd1 conditional mutants. (A) Longitudinal section of a wt primary cilium growing into a ciliary pocket. The ciliary shaft shows microtubular doublets orderly arranged. The basal body (bb) is visible at the base of the cilium. (B) Cross-section through the basal region of the ciliary shaft of a wt mouse primary cilium emerging from the ciliary pocket (p). The 9 microtubular doublets of the axoneme (ax) are orderly arrayed and devoid of dynein arms. (C) Longitudinal section of the ciliary shaft of primary cilia from Ofd1fl/+|Prx1Cre female in which some microtubular doublets are missing. (D) Cross-section through the proximal region of the ciliary shaft of Ofd1fl/+|Prx1Cre female primary cilium in which some microtubular doublets are missing (asterisks); only 7 microtubular elements are visible. (E) Cross-section through the distal region of the ciliary shaft of a Ofd1fl/+|Prx1Cre female primary cilium. In both panels D and E, the microtubular doublets are fewer than 9. (F) Longitudinal section of a primary cilium from Ofd1fl/+|Prx1Cre mutant within an invagination of the cell border at the beginning of ciliogenesis. A long basal body (bb) is observed. (G) Cross-section through the basal ciliary shaft shows an incomplete axoneme in which only 8 microtubular doublets, not orderly arranged, can be observed. (H, I) Cross-sections through the distal end of the ciliary shaft show few microtubular doublets and singlets in random position. Scale bars: 0.5 μm (A, C, E, F), 0.25 μm (B, D, G, H, I).
doublets are missing (Fig. 3C). The centrioles presented the conventional appearance and the basal bodies took a variable position close to the cell surface. A total of thirty primary cilia were observed on the twelve female mutants analyzed, sixteen displayed a pattern similar to that observed in the wt, and fourteen revealed to be shorter cilia (1.2 μm long) with aberrant anaxes. Such axones displayed a reduction in the number of microtubular doublets as shown in cross-sections at different levels of the ciliary shaft (Fig. 3D–E). Longitudinal sections of the ciliary anaxone confirmed that some doublets were missing (Fig. 3C). These differences were even more dramatic in the forelimbs of Ofd1fl/+Prx1Cre mutants. We observed the presence of defective primary cilia in twelve mutants. These cilia showed a rudimentary ciliary shaft emerging from the ciliary pocket (Fig. 3F). A long basal body was evident in the cytoplasm beneath the cillum. Fewer (from 6 to 8) microtubular doublets were observed in a dense material (Fig. 3G–I) in a few cases where the ciliary shaft appeared in its entire length. Only centrioles with an apparent normal structure were visible in most males (data not shown). Altogether these data indicate that Ofd1 participates in the mechanism of assembly and maintenance of the primary cilia anaxone in the limb bud mesenchyme.

Shh signaling is defective in Ofd1fl/+Prx1Cre mutants

Shh signaling plays an important role in digit formation and is required to maintain the expression of posterior genes (Chiang et al., 2001; Kraus et al., 2001). Polydactyly in mouse mutants has been associated with an anterior ectopic expression of Shh (Masuya et al., 1997), with a Shh-independent ectopic expression of Ihh and with a Shh-independent perturbation of Gli3 (Littingung et al., 2002; Masuya et al., 1997), an effector of the Shh signaling that acts as a transcriptional activator or, when cleaved, as a transcriptional repressor (Gli3R). To evaluate whether the polydactyly phenotype observed in Ofd1fl/+Prx1Cre mutants is associated with Hh signaling, we examined the pattern of expression of Shh, Ptc1 and Gli1 (Hui and Joyner, 1993). These genes are normally expressed in mutant limbs at E10.5 (Fig. S5B,C,E,F,H,I). At 48 somite stage (E11.5), while Shh was expressed normally (Fig. 4A–C), the expression of Ptc1 and Gli1 was found to be downregulated in Ofd1fl/+|Prx1Cre (Fig. 4E, H) and nearly absent in Ofd1fl/+|Prx1Cre male mutants (asterisks in Fig. 4F, I). We performed this experiment on the three different wt and mutant embryos. These data were also confirmed by real-time PCR experiment on forelimb of wt and mutants (Fig. 4J). Our analysis revealed the presence of 80%, and this indicates a progressive loss of Shh signaling after E10.5 despite the normal expression of Shh. We examined the expression pattern of downstream targets of Gli3 by whole-mount RNA in situ on mutant limb buds at E10.5 and E11.5. Hoxd12, Hoxd13, Greml1, and Hand2 are normally expressed in mutant limbs at E10.5 (data not shown) as expected for a weak knockout for Ofd1 at this stage. Interestingly at E11.5, the domains of expression of Hoxd12, Hoxd13, Greml1, and Hand2, which are normally expressed in the posterior region, were clearly expanded into the anterior part of the mutant male forelimbs (Fig. S6C, F, I, L). In the Ofd1fl/+|Prx1Cre female embryos, patches of cells ectopically expressing these transcripts were observed in the anterior half of the forelimbs (asterisks in Fig. S6B, E, H, K). X inactivation of the Ofd1 allele could account for the patchy ectopic expression in the female mutants, because only the cells with an inactivated wt allele would ectopically activate the genes.

To further exclude a possible role of Ofd1 transcript in the development of the AER and ectoderm, we analyzed the expression pattern of Fgf8, Bmp4, and Fgf4, which are key players in the normal development of these limb compartments. In situ hybridization analysis at E10.5 and E11.5 for Fgf8, Bmp4, and Fgf4 revealed a normal pattern expression on forelimbs of conditional mutants and wt, indicating that the AER and Fgfsignaling are not affected in Ofd1 conditional mutants (Fig. S7B, C, E, F, H, I and data not shown). These results indicate that Ofd1 inactivation in limb mesenchyme leads to progressive lack of Shh signaling and de-repression of posterior genes. Altogether our data indicate that Ofd1 should be considered a patterning factor involved in the correct determination of the A–P axis.

To assess the processing of Gli3, we performed Western blot analysis on protein lysates from limbs of wild-type and mutant embryos at E11.5, using an antibody against the N-terminus of Gli3 that recognizes both Gli3 activator (Gli3A) and repressor (Gli3R) forms (Chen et al., 2004; Wang et al., 2000). This analysis revealed that the amount of Gli3A was reduced, while Gli3R was dramatically increased in the limbs of mutant animals. As expected, the reduction of the repressor form and the increase of the activator form were more evident in the protein extracts from forelimbs of male mutants (Fig. 5A). As a result, the ratio of Gli3A versus Gli3R changed from approximately 0.25 (wt) to 2.7 in the forelimbs of Ofd1fl/+|Prx1Cre mutants (Fig. 5B). Moreover, the results obtained with Ptc1 and Gli1, which were strongly downregulated (Fig. 4F, I), demonstrate that there is also a reduction of Gli3R activity.

Ofd1fl/+Prx1Cre mutants display an abnormal endochondral bone development and defective Ihh signaling

The appendicular skeletal elements of Ofd1fl/+Prx1Cre mutants displayed a severe reduction in length (Fig. 11–L). Skeletal limb elements stained with Alcian Blue and Alizarin Red were used to quantify the reduction of the central ossified diaphysis of long bones in conditional mutants in comparison with wt at P0. This analysis confirmed that the forelimbs were more affected than the hindlimbs. The radius was the most affected bone, with a 64% reduction in length in male mutants and a 31.8% reduction in female mutants in comparison with wt animals (Table 1). Also in Ofd1fl/+|Prx1Cre mutants, the radius was the most affected bone with a reduction in length of 47% in female mutants (data not shown), Hematoxylin and eosin staining on E16.5 sections of mutant forelimbs demonstrated shortening of long bones suggesting a defect in endochondral bone formation (Fig. 6A–C). The growth plate of appendicular long bones appeared to be reduced in size and highly disorganized as shown for the ulna (Fig. 6D–F). Similar results were obtained for humerus and radius. Reduction of bone mineralization is a constant phenotype of Ofd1fl/+Prx1Cre animals, indicating a defective formation of the bone collar. Von Kossa staining was performed at E16.5 to study the matrix mineralization (Fig. 6G–L). This study revealed a reduction of mineralization, which was more evident in Ofd1fl/+|Prx1Cre mutants. In these mice, the bone collar surrounding the hypertrophic chondrocytes could barely be detected (asterisks in Fig. 6L). Histological staining did not show the presence of ectopic perichondrial cells (data not shown).

Ihh controls multiple aspects of endochondral skeletal development, including proliferation and differentiation of chondrocytes, osteoblast development, and vascularization (Olsen et al., 2000). We therefore analyzed the expression of Ihh in Ofd1fl/+Prx1Cre mutants at E16.5. At this stage, Ihh is expressed in the prehypertrophic and early hypertrophic chondrocytes in the growth plate. Our results showed that Ihh was normally expressed in Ofd1fl/+Prx1Cre conditional mutants (Fig. 7B, C). Ptc1 and Gli1, which are downstream targets of the Ihh pathway commonly used as readout of Ihh activity, are expressed in the columnar and early hypertrophic chondrocytes, and in the perichondrium. RNA in situ studies revealed that the expression of Ptc1 and Gli1 was drastically reduced in Ofd1fl/+Prx1Cre mice (Fig. 7E, F, H, I), and in particular, the expression of these molecules was barely detectable in the perichondrium of male mutants (asterisks in Fig. 7F, I), providing evidence of defective Ihh signaling. Shortened long bones could result from decreased proliferation of chondrocytes, increased apoptosis, and/or premature differentiation either of distal to columnar or of columnar to hypertrophic chondrocytes. To test whether proliferation was affected, we performed in vivo a short pulse BrdU labeling experiment followed by immunohistochemistry (Fig. 7J–L). A significant decrease in the percentage of BrdU-positive chondrocytes was observed in the proximal ulna of Ofd1fl/+|Prx1Cre mice at E16.5 (Fig. 7M). This result indicates a
reduction of chondrocytes proliferation probably due to defective Ihh activity. To complete the study, we also analyzed the amount of apoptosis by TUNEL assays and no differences were observed between wt and Ofd1 conditional mutants (data not shown). We finally studied the expression pattern of Pthrp, which is regulated by Ihh signaling and plays a key role in chondrocyte maturation (Kronenberg, 2003). Pthrp expression was decreased in Ofd1fl/Prx1Cre mice (Fig. 8B) and almost absent in Ofd1fl/y|Prx1Cre (Fig. 8C). We also analyzed PPR, the Pth/Pthrp receptor, which is normally expressed in the prehypertrophic chondrocytes and in the osteoprogenitor cells of the perichondrium (arrows in Fig. 8D). Our study revealed that PPR, which is normally expressed in the prehypertrophic chondrocytes of Ofd1 mutant animals, was reduced in the perichondrium of mutant females (arrows in Fig. 8E) and absent in the perichondrium of Ofd1fl/y|Prx1Cre mutants (asterisks in Fig. 8F), consistent with the mineralization defects detected by the Von Kossa staining (Fig. 6). In order to test whether the growth plate defects observed in Ofd1 mutant animals were due to premature differentiation of chondrocytes, we also analyzed the expression of Fgfr1, a marker of

Fig. 4. Shh signaling is defective in Ofd1fl/Prx1Cre mutant limbs at E11.5. Whole-mount in situ hybridization analysis at E11.5 shows that Shh expression is normal in Ofd1fl/Prx1Cre mutant limbs (B–C) compared to wt (A). Ptch1 and Gli1 are expressed in cells adjacent the Shh expression domain in wt forelimbs (D, G). Expression of Ptch1 and Gli1 is downregulated in Ofd1fl/Prx1Cre (E, H) and almost absent in Ofd1fl/y|Prx1Cre (asterisks in F, I). Forelimbs are viewed dorsally. (J) Quantitative RT-PCR with primers for Ptch1, Gli1, and Shh at E11.5. The analysis was performed on RNA from forelimbs of conditional mutants (lanes 2, 3, 5, 6, 8, 9 in J) and wt (lanes 1, 4, 7 in J). Significant differences between mutants and controls (p<0.05) are indicated (asterisk). Error bars represent standard error of the mean.
of wt and mutants, respectively. The results were obtained from four independent experiments. Abbreviations:

Discussion

Ciliary dysfunction is associated to limb and skeletal abnormalities observed in Ofd1 mutants

OFD1 is a centrosomal/basal body protein localized at the origin of primary cilia in different cell types (Giorgio et al., 2007; Romio et al., 2004). We previously demonstrated that early inactivation of the Ofd1 transcript is associated with the lack of formation of cilia in the embryonic node of male mutants, leading to abnormalities in left–right axis specification (Ferrante et al., 2006). Several experimental findings link limb abnormalities to dysfunction of primary cilia. Moreover, intraflagellar transport and primary cilia have also been implicated in skeletal development suggesting that defective primary cilia affect the organization of the growth plate (McGlashan et al., 2007; Serra, 2008; Song et al., 2007). Very recently, a cytoplasmic dynein, involved in retrograde transport to the cilium, has been shown to be mutated in short-rib-polydactyly syndrome with affected patients displaying abnormal cilia (Dagoneau et al., 2009; Merrill et al., 2009).

We here show that conditional inactivation of Ofd1 in limb mesenchyme results in severe polydactyly with loss of digit identity in the presence of shorter and malformed cilia at E11.5, in both null and conditional mutants. In addition, immunofluorescence analysis also demonstrated the presence of shorter cilia on chondrocytes at E16.5 in Ofd1 mutants (data not shown) and it is conceivable to hypothesize a possible role of primary cilia in Ihh signaling although further experiments will be needed to address this issue. A number of different mutants for proteins involved in ciliary function displaying limb and skeletal abnormalities have been reported to date (Haycraft and Serra, 2008). However, it remains unclear as to which features are directly linked to abnormal cilia, resulting in a defective Shh signaling, and which features are due to a direct role of ciliary proteins in limb and skeletal development.

Interestingly, Ofd1 seems to be essential for the appearance of cilia in the embryonic node (Ferrante et al., 2006) and in mouse embryonic stem cells (Corbit et al., 2008), whereas primary cilia do appear, although malformed, in the developing limb bud. Primary cilia are present on almost all mammalian cells and although recent data have shed light on their multiple roles, the full meaning of their functions in different cell types remains poorly understood. It is conceivable to hypothesize that diverse phenotypes caused by ciliary dysfunction may reflect variable expression and functions of ciliary proteins in different cell and tissue types (Bisgrove and Yost, 2006).

Comparison of Ofd1fl+/Prx1Cre with Ift mutants

Defects in IFT and Kif3a cause embryonic lethality, and it is only with the generation of mice carrying a conditional Ift88 and Kif3a allele and the Prx1Cre transgene that it has been possible to study endochondral bone formation in IFT mutants (Haycraft et al., 2007). Ofd1fl+/Prx1Cre, the Prx1Cre;Ift88, and the Prx1Cre;Kif3a conditional mice display a very similar phenotype characterized by severe polydactyly and shortened long bones. They have decreased Shh signaling after E10.5, a decreased

Table 1

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<tr>
<th>Forelimbs</th>
<th>Hindlimbs</th>
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<tr>
<td><strong>Humerus (%)</strong></td>
<td><strong>Femur (%)</strong></td>
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<tr>
<td>WT n = 8</td>
<td>100 ± 0.14</td>
</tr>
<tr>
<td>Oft1fl+/Prx1Cre n = 8</td>
<td>84.6 ± 0.24</td>
</tr>
<tr>
<td>Oft1fl+/Prx1Cre n = 8</td>
<td>46.8 ± 0.20</td>
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<tr>
<th><strong>Ulna (%)</strong></th>
<th><strong>Tibia (%)</strong></th>
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<tr>
<td>100 ± 0.14</td>
<td>100 ± 0.16</td>
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<tr>
<td>84.4 ± 0.12</td>
<td>77.9 ± 0.12</td>
</tr>
<tr>
<td>51.3 ± 0.22</td>
<td>51.0 ± 0.14</td>
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<tr>
<th><strong>Radius (%)</strong></th>
<th><strong>Fibula (%)</strong></th>
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<tr>
<td>100 ± 0.20</td>
<td>100 ± 0.20</td>
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<tr>
<td>68.2 ± 0.23</td>
<td>82.6 ± 0.07</td>
</tr>
<tr>
<td>36.0 ± 0.14</td>
<td>50.0 ± 0.17</td>
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Average length of wt was set to 100%. Mutant and control limbs were collected from eight litters.
activation of Ihh targets Ptc1 and Gli1 in the growth plates of long bones and delayed bone collar formation. A difference between Ift and Ofd1 mutants relates to Ihh expression, which appears normal in Ofd1 mice but is reduced in the Prx1Cre conditional allele of Ift88 and Kif3a. In addition, Pthrp expression is normal in the Prx1Cre conditional allele of Ift88 and Kif3a, while is reduced in Ofd1/Prx1Cre mice. Chondrogenic clumps of cells surrounding the perichondrium are described in Prx1Cre mice. Ihh conditional mice, this feature has not observed in the Ofd1 mutants. Moreover, the main difference between Ift and Ofd1 conditional mutants relates to cilia, which are shorter and malformed in limb and chondrocytes of Ofd1 mice but are absent in the limb and chondrocytes of the Prx1Cre conditional allele of Ift88 and Kif3a (Haycraft et al., 2007). This evidence supports the idea that Ofd1 acts to regulate cilia function in the limb even though cilia are still present after Ofd1 deletion.
Shh signaling is defective in Ofl1 mutants

Primary cilia play an important role in mediating Hh signaling. Ptc1 localizes to primary cilia, inhibits the Hh pathway, and allows cilia to function as chemosensors for the detection of extracellular Shh (Rohatgi et al., 2007). In addition, mobilization of Smo to primary cilia has been shown to be a key event in the activation of the Shh pathway (Corbit et al., 2005). Finally, Sufu and the three full-length Gli transcription factors also localize to cilia (Corbit et al., 2005; Haycraft et al., 2007; May et al., 2005).

Ofl1 conditional mutants displayed extensive polydactyly with loss of anterior/posterior (A/P) patterning. The A/P patterning is determined by the balance between Shh, expressed in the zone of polarizing activity, and Gli3, one of the main transducers of the Hh signaling (Tickle, 2006). Gli3 exists as a full-length activator (Gli3A) that is proteolytically processed into a Gli3 repressor (Gli3R) form in the absence of the Shh ligand (Wang et al., 2000). Shh promotes the development of five patterned digits by inhibition of the proteolytic processing of Gli3 to generate the Gli3 repressor gradient along the A/P axis of the limb bud. This causes the expansion of...
posterior genes in the anterior limb mesenchyme (Litingtung et al., 2002; te Welscher et al., 2002). We showed that the polydactyly and the unpatterned digits displayed by Ofd1 conditional mutants were associated with a defective Shh signaling demonstrated by the dowregulation of the target genes Gli1 and Ptc1 at E11.5 and to an increased ratio of Gli3A versus Gli3R, as has been previously suggested for mutants associated with ciliary dysfunction (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Vierkotten et al., 2007). This result supports the observation that the ratio of the Gli3 activator to repressor is more important than the Gli3 repressor gradient in the determination of digit number and identity (Wang et al., 2007).

Null Ofd1 mice display a normal pattern of expression of Gli1 and Ptc1 in limbs at E10.5 (Ferrante et al., 2006). However, we cannot exclude the possibility that Ofd1 null mice also display a dowregulation of Gli1 and Ptc1 at E11.5, as we demonstrated in the Ofd1 conditional mice. This cannot be assessed consistently in null mice, because the Ofd1 males are severely damaged by E11.5 (Ferrante et al., 2006). Probably the deregulation of Shh signaling in Ofd1 mutant limbs starts after E10.5, and this explains the fact that in Ofd1 null mice the expression of Gli1 and Ptc1 appears to be normal at E10.5 (Ferrante et al., 2006). We do plan to address this issue, when we examine in more detail the role of Ofd1 in Shh signaling during limb development in future studies.

It is tempting to speculate that the cilia abnormalities observed in Ofd1 mutants (see below) may affect the normal localization of components of the Ihh signaling and in particular of Gli3A at the distal tip of cilia, leading to a defective processing of Gli3 and an impaired transduction of Shh signaling. As opposed to Ift mutants, Ofd1 mutants do have cilia, although abnormal, which make them valuable tools to study the role of primary cilia in the mechanisms underlying Shh signaling and the processing of Gli3.

**Ofd1 is involved in endochondral bone development**

Endochondral ossification involves the formation of a cartilage primordium and a growth plate, where chondrocytes initially undergo proliferation and where a series of differentiation steps take place secreting a cartilage template that is eventually replaced by bones (Olsen et al., 2000). Ihh is a key regulator of chondrocytes proliferation and differentiation. In Ihh null mice, chondrocytes showed reduced proliferation, premature hypertrophy and loss of Pthrp expression in the articular cartilage and in the perichondrium, leading to shortened long bones (Karp et al., 2000). Previous studies have shown which features of the Ihh/−/− phenotype result from elevated Gli3R levels and which from decreased Gli3A functions. In particular, defective bone collar formation and absence of Gli1 and Pthrp expression were shown to be due to impaired Gli3A functions, while the abnormalities in chondrocytes proliferation observed in the growth plate of Ihh/−/− mice were associated to increased Gli3R activity (Hilton et al., 2005; Koziel et al., 2005). A model was proposed in which the Ihh/Gli3 system regulates two distinct steps of chondrocyte differentiation: (1) the switch from distal into columnar chondrocytes, which is repressed by Gli3 using a Pthrp-independent mechanism, and (2) the transition from columnar into hypertrophic chondrocytes, which is regulated by Gli3-dependent expression of Pthrp (Koziel et al., 2005). In this study, we demonstrated that Ofd1 is required for normal Ihh signaling activity based on the loss...
of Pcth1 and Gli1 expression in the long bones of Ofd1 mutant limbs. We also observed a defective formation of the bone collar and a reduced expression of Pthrp. These features are probably associated with impaired Gli3 response activity as observed by defective Gli3 processing and reduced expression of Gli1 and Pcth1 in the limb buds at E11.5. However, we also observed defective proliferation of chondrocytes not associated with an increased activity of Gli3. Moreover, the transition of distal to columnar chondrocytes, which is directly regulated by Gli3, was normal in Ofd1fl/flPrx1Cre mutants. On the basis of these observations, we postulate that Ofd1fl/flPrx1Cre mutants probably lack the Ihh-dependent Gli3 activator functions during growth plate development and that the shortening of long bones is essentially due to premature differentiation of hypertrophic chondrocytes and defective mineralization. The presence of an “inactive” Gli3 would explain the delay in the Pthrp-mediated transition of columnar to hypertrophic chondrocytes. However, the reduction of Gli3 cannot explain the defects in chondrocytes proliferation and the normal progression from distal to columnar chondrocytes observed in Ofd1 conditional mutants. Therefore, Ofd1 may also play a role in regulating the expression of Pthrp. Analysis of Ofd1 and ciliary function during long bone development was previously unattainable due to the early lethality of Ofd1+/- male mutants. The use of these conditional cilia-Ofd1 mutant alleles in combination with additional strains expressing Cre recombinase under the control of alternative promoters will allow a more thorough analysis of the functional importance of Ofd1 and cilia throughout development and during postnatal life.

Conclusions

We developed a new mouse model with limb-specific Ofd1 inactivation, which reproduces the limb abnormalities observed in oral–facial–digital type 1 syndrome. Conditional inactivation using different Cre lines allowed us to study the role of the Ofd1 transcript in the limb bud and to demonstrate that this role is specific to the mesoderm. Ofd1 mutant animals displayed extensive polydactyly with loss of anterior-posterior patterning and shortening of long bones. Characterization of these mutants revealed that Ofd1 plays a critical role in A/P patterning and that the shortening of long bones is associated to the reduction in proliferation, disorganization of the columnar chondrocyte, defective bone collar formation. Immunofluorescence and ultrastructural studies allowed us to demonstrate that Ofd1 is necessary for correct chondrogenesis in the limb bud. These data increase our insight into how ciliary proteins may exert their function in limb development and endochondral ossification.

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Appendix A. Supplementary data

Supplementary materials related to this article can be found online at doi:10.1016/j.ydbio.2010.09.020.

References


