Knockdown of the Intraflagellar Transport Protein IFT46 Stimulates Selective Gene Expression in Mouse Chondrocytes and Affects Early Development in Zebrafish*

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Bone morphogenetic proteins (BMPs) act as multifunctional regulators in morphogenesis during development. In particular they play a determinant role in the formation of cartilage molds and their replacement by bone during endochondral ossification. In cell culture, BMP-2 favors chondrogenic expression and promotes hypertrophic maturation of chondrocytes. In mouse chondrocytes we have identified a BMP-2-sensitive gene encoding a protein of 301 amino acids. This protein, named mIFT46, is the mouse ortholog of recently identified Caenorhabditis elegans and Chlamydomonas reinhardtii intraflagellar transport (IFT) proteins. After generation of a polyclonal antibody against mIFT46, we showed for the first time that the endogenous protein is located in the primary cilium of chondrocytes. We also found that mIFT46 is preferentially expressed in early hypertrophic chondrocytes located in the growth plate. Additionally, mIFT46 knockdown by small interfering RNA oligonucleotides in cultured chondrocytes specifically stimulated the expression of several genes related to skeletogenesis. Furthermore, Northern blotting analysis indicated that mIFT46 is also expressed before chondrogenesis in embryonic mouse development, suggesting that the role of mIFT46 might not be restricted to cartilage. To explore the role of IFT46 during early development, we injected antisense morpholino oligonucleotides in Danio rerio embryos to reduce zebrafish IFT46 protein (zIFT46) synthesis. Dramatic defects in embryonic development such as a dorsalization and a tail duplication were observed. Thus our results taken together indicate that the ciliary protein IFT46 has a specific function in chondrocytes and is also essential for normal development of vertebrates.

In vertebrates endochondral bone formation involves first the formation of cartilage primordia, followed by their replacement by bone. This process is associated with several changes in the differentiation status of chondrocytes in the growth plate and results in a modification of the extracellular matrix composition. The cartilage matrix contains predominantly type II collagen. During endochondral ossification, chondrocytes enter a process of maturation characterized by cellular hypertrophy, a decrease in type II collagen expression, and the onset in type X collagen expression. After full differentiation of hypertrophic chondrocytes the cartilage matrix calcifies. Ossification begins with the invasion of the calcified cartilage by blood capillaries. This neo-vascularization of the growth plate is concomitant with apoptosis of most hypertrophic chondrocytes. The resulting cartilage matrix provides a scaffold for osteoblasts, which invade the cartilage mold, along with blood vessels, so laying down a bone matrix characterized by the presence of osteocalcin (reviewed in Ref. 1).

The successive steps of chondrocyte differentiation are finely regulated by different signaling molecules. Several cytokine families are involved in cartilage development such as Hedgehog (Hh), transforming growth factor-β (TGFβ), or Wnt. These signals coordinate proliferation, hypertrophy, and extracellular matrix protein production to maintain growth plate homeostasis and function. Among the members of the transforming growth factor-β superfamily, bone morphogenetic proteins (BMPs) play an essential role not only during the formation of cartilage molds for long bones, vertebrae, and ribs, but also in their transition to bone during endochondral ossification (reviewed in Ref. 2). BMPs are also involved in other important physiological events such as dorsoventral determination during gastrulation in early embryogenesis. Originally BMPs were described for their ability to induce ectopic bone formation by reproducing the events occurring during endochondral ossification (3). In fact, recent experiments have shown that only a few BMP members can induce de novo bone formation in the classic subcutaneous implantation assay (4–8) and potentiate chondrocyte and osteoblast differentiation in vitro (9–14). We previously reported that BMP-2 and BMP-4, but not BMP-12 or BMP-13, can stimulate chondrogenic expression, hypertrophic maturation of chondrocytes, and their osteoblastic differentiation (15, 16). BMP-2 is also able to reverse the program of chondrocyte de-differentiation induced in cell culture, as attested by re-expression of the

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cartilage-characteristic type IIB procollagen pre-mRNA (17). Thus BMP-2 is a strong anabolic agent with regard to cartilage biology. The fact that *bmp-2* mRNA is expressed in hypertrophic chondrocytes of the growth plate in mouse embryos (18) strongly suggests that BMP-2 plays a physiological role during endochondral ossification.

As an approach to attaining better understanding of the molecular mechanisms involved in chondrocyte differentiation and maturation, we examined differentially expressed genes in mouse chondrocytes cultured in the presence or absence of BMP-2, by using the differential display reverse transcription-PCR technique. We isolated a gene encoding the mouse ortholog of an intraflagellar transport (IFT) protein, IFT46, recently identified in *Caenorhabditis elegans* (19) and *Chlamydomonas reinhardtii* (20). We have termed this gene *mIFT46* (mouse IFT46) and showed that its expression is up-regulated in mouse chondrocytes upon BMP-2 treatment and that endogenous *mIFT46* protein is located in the primary cilium in mouse chondrocytes. In situ hybridization analyses in mouse embryo limb buds revealed that *mIFT46* is expressed in cartilage undergoing endochondral ossification. Interestingly, the siRNA-mediated silencing of *mIFT46* mRNA in primary chondrocytes modulated the expression of certain genes related to skeletogenesis. Additionally Northern blotting analysis indicated that *mIFT46* is also expressed well before chondrogenesis during embryonic mouse development. To explore the role of IFT46 during early development, we used antisense morpholinos to deplete the zebrafish ortholog of IFT46 protein (*zIFT46*). Firstly, we found that *zIFT46* is expressed at a very early stage of zebrafish development. Secondly, we observed morphological defects in embryos, such as dorsalization and tail duplication, as a consequence of *zIFT46* knockdown. Thus our results taken together indicate that the ciliary protein IFT46 has a specific function both in chondrocytes and during the early development of vertebrates.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The MC615 cell line was characterized previously (21). For the studies with freshly isolated chondrocytes, embryonic mouse chondrocytes were extracted from the ventral part of the rib cages of 17.5 days post coitum mice, as described (22). The MC615 cells and rib chondrocytes were maintained in 1:1 high-glucose Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% FBS and supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (all products from Amersham Biosciences—Invitrogen) on Corning dishes. Cultured rib chondrocytes were designated P0 after their isolation and P1 after their first passage. For the different experiments, MC615 cells and embryonic rib chondrocytes were grown for the indicated days in 1% FBS-supplemented medium and treated with BMP-2 (0–400 ng/ml). Recombinant human BMP-2 was produced and purified by Wyeth (Cambridge, MA). The culture medium supplemented or not with BMP-2 was replaced each day.

**Zebrafish**—Zebrafishes (a cross between AB and Tübingen) were maintained under standard laboratory conditions (23).

**Morpholino and mRNA Microinjections**—Morpholinos (labeled with fluorescein) were designed according to the manufacturer’s recommendations (Gene Tools): *zIFT46*-MO (5’-GGTGCTCTGTTGCTGCCCGTC-3’) hybridizes with *zIFT46* mRNA from position 25 to 10 relative to the start codon. We used the same sequence with five mismatches (underlined) as negative control: 5mis-MO (5’-GGTCTCTGTTGCTGCCCGTC-3’). Morpholinos were injected (5–10 nl) into 1–4 cell stage embryos at 1 mm concentration in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES, pH 7.6). For rescue experiments, full-length *zIFT46* cDNA was amplified by RT-PCR from total RNA isolated from zebrafish embryos using the following sense (5’-ATGATGGGGATCACCAGTCAATGTTAGCT-3’) and antisense (5’-ATGATGGCTCGAGTCCTCCAGATGCTAGTTGT-3’) primers and then cloned between BamHI and Xhol restriction sites of the pCS2+ vector for in vitro transcription (SP6 mMESSAGE mMACHINE™ kit, Ambion). After NotI linearization, the reaction was performed using 1 μg of plasmid template as indicated by the manufacturers. RNAs were microinjected at a 100 ng/μl concentration in nuclease-free water.

**RNA Isolation**—Total RNAs from MC615 cells and embryonic rib chondrocytes were extracted using the RNeasy mini kit (Qiaogen) as recommended by the manufacturer. Total RNAs were isolated from (i) rib cages of 2-day-old-mice, (ii) 8.5–16.5 days post coitum mouse embryos, and (iii) different mouse adult tissues, as described previously (24). Briefly, embryos or tissues were frozen in liquid nitrogen, powdered with a mortar and pestle, homogenized in lysis buffer containing 4 M guanidine isothiocyanate, 25 mM sodium citrate, 0.5% N-laurylsarcosine, and 0.1% β-mercaptoethanol. After phenol-chloroform extraction and precipitation, pellets were treated with 200 μg/ml proteinase K until complete dissolution. RNA was then purified by ultracentrifugation on a cesium trifluoroacetate cushion. Total RNAs from zebrafish embryos were extracted at different developmental stages using a similar method except that RNAs were not treated with proteinase K and were purified in a cesium chloride gradient (25).

**Differential Display RT-PCR**—PCR-based differential screening was performed essentially as previously described (24, 26). Total RNAs extracted from MC615 cells were digested with RQ1 RNase-free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA. For RT, a 40-μl reaction contained 500 ng of total RNA, 1 μM oligo(dT12VN) primers (V represents A, G, or C and N represents A, T, G, or C), 10 mM dithiothreitol, 4 units of RNasin (Promega), 0.1 mg/ml bovine serum albumin, and 320 μM dNTPs. Samples were incubated at 65°C for 5 min then at 42°C for 5 min prior to addition of 400 units of SuperScript II-RNase H− (Invitrogen) for 50 min at 42°C. The reverse transcriptase was then heat-inactivated for 15 min at 70°C. For PCR amplification, each 20-μl reaction contained a 2 μl of RT aliquot. Different subsets of oligo(dT12VN) and arbitrary primers were tested to enrich the diversity of differentially expressed mRNA (27). Briefly, PCR reactions were performed with 5 units of AmpliTag® DNA polymerase (Applied Biosystems) in the presence of 0.2 μM oligo(dT12VN) primers, 0.2 μM arbitrary primers, 2 μM dNTPs, and 1 μl of [α-35S]dATP (PerkinElmer Life Sciences, 1200 Ci/mmol). Amplifications were performed in a GeneAmp 2400 PCR System (Applied Biosystems). Following an initial
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denaturation step of 5 min at 95°C, amplification consisted of 40 cycles of 45 s at 95°C, 2 min at 42°C, 30 s at 72°C, followed by a final extension step of 5 min at 72°C. PCR products were separated on a 6% DNA sequencing gel, which was exposed for autoradiography after drying. The selected bands were excised from the gel, and DNA was eluted. The eluted cDNAs were re-amplified by PCR by using the same subset of oligo(dT12 VN) and arbitrary primers, cloned into the pCR® 2.1-TOPO vector according to the recommendations of the manufacturer (Invitrogen) and sequenced (Genome Express SA, Meylan, France). The mIFT46 sequence was obtained using dT12 Vc and DD57 primers (Table 1).

Conventional and Quantitative RT-PCR—Total RNAs were treated with RQ1 RNase-free DNase (Promega). Conventional RT-PCR were performed as previously described (16) using specific primers designed according to sequences available in the databases or published by other authors (Table 1). Primers for mouse glyceraldehyde-3-phosphate dehydrogenase (Gapdh) were used to ascertain if an equivalent amount of cDNA was synthesized. In parallel, controls without reverse transcriptase were performed to demonstrate the specificity of the reactions (data not shown).

Quantitative PCR were performed using the iQ SYBR Green PCR Supermix (Bio-Rad) in a total volume of 20 µl with 4 µl of 10-fold-diluted cDNA, in the presence of 300 nM of specific primers designed according to sequences available in the databases or published by other authors (Table 1). Levels of gene expression in each sample were determined by using the comparative C_t method with Gapdh and zHistone 2A genes as endogenous controls for mouse cells and zebrafish embryos, respectively.

Northern Blot Analysis—Equal amounts (20 µg) of total RNAs were size-fractionated by electrophoresis through 1% agarose-formamide gels and transferred to nylon N-membranes (Amersham Biosciences). Northern hybridization was carried out as previously described (16). A 462-bp EcoRI-XhoI restriction fragment corresponding to mIFT46 cDNA was used as a probe.

In Situ Hybridization—In situ hybridizations on limb sections of 17.5-day post coitum mice were performed as described (29, 30) using DNA probes labeled with [α-32P]dCTP by random priming. Probes corresponding to mouse cDNAs encoding α1(II) procollagen and α1(X) procollagen were previously described (16). A 445-bp PvuII restriction fragment of the p Bluescript SK+ plasmid was routinely used as a control probe for nonspecific hybridization (data not shown).

Whole mount in situ hybridizations in zebrafish embryos were performed using antisense and sense (as a negative control) digoxigenin-labeled Riboprobes, as described before (31). To generate a zIFT46 DNA probe, a zIFT46 cDNA sequence was amplified by RT-PCR from total RNA isolated from zebrafish embryos. The amplicon was then cloned into a pBlueScript SK+ plasmid to synthesize Riboprobes. The zCol2a1 and the zeve1 Riboprobes were gifts of Dr. Y. Yan (University of Oregon) and Dr. B. Thiese (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Strasbourg, France), respectively.

siRNA and PCR Array Analysis—Two siRNA oligonucleotides for mIFT46 were purchased from Dharmacon Inc. (Lafayette, CO): siRNA IFT46 #1 (5'-UCAAGAACUCCGCAUUUU-3') and siRNA IFT46 #2 (5'-CGUAGUCCUGCCGCAUUU-3'). The siRNAs were independently transfected into P1 chondrocytes by using the Human Chondrocyte Nucleofector kit (Amaxa, Cologne, Germany) according to the manufacturer’s protocol (Program U-24). Briefly, 3 × 10^6 cells were suspended in 100 µl of electroporation buffer with 250 nM of each siRNA. An siRNA directed against the green fluorescent protein (GFP) and provided with the siRNA test-kit (Amaxa) was used as control. After electroporation, cells were immediately transferred into 6-well plates in 1:1 high-glucose Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% FBS. After 24 h of culture, total RNAs were extracted as described above and were analyzed with the Agilent 2100 Bioanalyzer and the RNA 6000 Nano Assay kit (Agilent Technologies, Palo Alto, CA) to determine RNA quality and quantity. PCR array analysis was performed by using 450 ng of total RNA of each sample and the RT2 Profiler™ PCR array mouse osteogenesis (SuperArray, Bioscience Corp.), according to the recommendations of the manufacturer. Levels of gene expression were determined with the comparative C_t method. The -fold change of gene expression in mIFT46 knocked-down chondrocytes compared with control GFP siRNA-nucleofected chondrocytes was calculated with Microsoft Excel software. Candidate genes with -fold changes >3.5 were selected. Similar results were obtained using independently either IFT46 #1 (Fig. 7) or IFT46 #2 (data not shown) siRNAs.

Construction of Recombinant Adenovirus-transducing mIFT46—A recombinant adenovirus carrying a full-length cDNA encoding mIFT46 was constructed, amplified, and titrated by Genethon (Evry, France). Expression of mIFT46 was under control of the cytomegalovirus promoter. Mouse chondrocytes were infected with this adenovirus preparation as previously described (16).

Production and Purification of Recombinant IFT46 Protein—For the heterologous production of mIFT46 protein in bacteria, full-length mIFT46 cDNA was cloned into a pT7.7 vector harboring a His6 tag sequence between PstI and HindIII restriction sites. First, mIFT46 coding sequence was amplified by RT-PCR from total RNA extracted from mouse chondrocytes, by using the sense (5'-CATCATGTTGTCTC-3') and antisense (5'-AGTATGCTGCAGGATGAA-3') primers, and the amplicon was cloned into BamHI and PstI restriction sites of the pT7.7 vector. The resulting construct expresses C-terminal His-tagged mIFT46. Recombined plasmids were transformed into Escherichia coli BL21(DE3). The mIFT46 protein was induced with isopropyl-1-thio-β-D-galactopyranoside and purified from the insoluble fraction after solubilization in 8 M urea by metal chelating.
Preparation and Affinity Purification of Antibodies—Anti-IFT46 serum was prepared by immunizing rabbits (IUT A-Lyon 1, France) with the IFT46 recombinant protein. After ammonium sulfate precipitation, antibodies were affinity-purified over a column of IFT46, which was immobilized on CNBr-activated Sepharose (Amersham Biosciences).

Immunoprecipitation, Western Blot Analysis, and Immunofluorescence—Rib chondrocytes freshly isolated from 17.5-day post coitum mice were collected in lysis buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM NaF, 1 mM Na3VO4, 2 μg/ml leupeptin, 5 μg/ml pepstatin A, 2 μg/ml aprotinin, 1 mM N-ethylmaleimide, and 1 mM phenylmethylsulfonyl fluoride). The lysate was cleared by centrifugation, and protein concentration in the supernatant was measured by the BCA protein assay (Sigma). Equal amounts of protein (300 μg) were incubated for 90 min at 4 °C with 1 μg of antibodies to IFT46 or rabbit IgG. Protein-G-Sepharose beads (Amersham Biosciences) were added, and the mixtures were incubated at 4 °C for 75 min. The beads were washed twice with lysis buffer, once with TBS-T (20 mM Tris-HCl, 0.5 mM NaCl, pH 7.5, containing 0.1% Tween 20), and once with TBS (TBS-T without Tween). The immunoprecipitation products were dissolved in reducing SDS-PAGE sample buffer and run on 12% polyacrylamide gels. Immunoblots were probed with affinity-purified antibodies to IFT46 followed by goat alkaline phosphatase-conjugated anti-rabbit IgG. Bound antibodies were detected on x-ray films using a Bio-Rad Immun-star chemiluminescent substrate.

Indirect immunofluorescence detection was performed as previously described (17). Double staining was obtained using polyclonal anti-IFT46, monoclonal anti-acetylated α-tubulin antibodies (Sigma), and anti-rabbit IgG Cy2-coupled (Chemicon) anti-mouse IgG Cy3-coupled (Jackson ImmunoResearch) as secondary antibodies.

RESULTS

Identification of mIFT46 as a BMP-2-sensitive Gene in Chondrocytes—MC615 chondrocytes were cultured for 3 days in the presence of 1% FBS alone or supplemented with 50 or 100 ng/ml BMP-2 to modulate their phenotype. In these conditions we previously reported that BMP-2 favors chondrogenic expression and promotes hypertrophic maturation and osteoblastic-like differentiation of chondrocytes (16). Total RNA extracts from these cells were used for differential display PCR to identify BMP-2-responsive genes in MC615 cells. By using dT12VC and DD57 primers (Table 1), we isolated a cDNA fragment, which appeared only in the display of mRNA from MC615 cells treated with 50 or 100 ng/ml BMP-2 (data not shown). Sequencing of the corresponding fragment identified it as an mRNA encoding a hypothetical protein previously named ORF-2 (accession number AJ249981) and found by an expressed sequence tag based in silico gene cloning (32).

RT-PCR using specific primers derived from ORF-2 sequence confirmed that this gene is up-regulated by BMP-2 in MC615 chondrocytes (Fig. 1A) as well as in primary embryonic mouse chondrocytes (Fig. 1B). Total RNAs were also extracted from rib cage of 2-day-old mice. This tissue undergoes endochondral ossification and, therefore, was used as a positive control for RT-PCR analysis of gene expression characteristic of cartilage or bone cells. Indeed, this newly identified gene was expressed in the rib cage (Fig. 1A).

| Gene          | Primers Strain | Product size | Reference |
|---------------|----------------|--------------|-----------|
| Oligonucleotide primers used for DDRT-PCR | | | |
| DD-57         | 5’-GAGAAGCAGCT-’ | +          | 27        |
| dT12VC        | 5’-TTTTTTTTTTTTTCAC-’ | -          | 26        |
| Oligonucleotide primers used for conventional RT-PCR | | | |
| mIFT46        | 5’-ATGGCTAATATACACCTGGAC-’ | +          | 906       |
|              | 5’-TGAGTAAAGCTGCTGTTT-’ | -          | 17        |
| Col2a1        | 5’-ATCACTGTGACCCACAGAGGG-’ | +          | 463       |
| Sox9          | 5’-TTCGCGAGCTGGCCGGGAA-’ | -          | 11        |
| Col10a1       | 5’-GGAACATAGGCTAATTG-’ | +          | 596       |
| Gapdh         | 5’-AAGGATGCTGCCACCAACAGAG-’ | +          | 443       |
| Oligonucleotide primers used for quantitative RT-PCR | | | |
| mIFT46        | 5’-CCTCGAGGACTTGGCTTCAAAAT-’ | +          | 98        |
|              | 5’-GAGATGCTTGGCTTCAAAATG-’ | -          | 86        |
| Col2a1        | 5’-CACGAGATTCCCCCCTTAC-’ | +          | 140       |
| Col10a1       | 5’-GAGATGCTATTTGGAAGTG-’ | -          | 132       |
| Gapdh         | 5’-AATCTGGCACTTGGCTTTC-’ | +          | 131       |
| zIFT46        | 5’-ATCTGGCAACTCAGCCTCCCTT-’ | -          | 247       |
| zHistone 2A   | 5’-TCTCGAGATTCCCCTGGA-’ | +          | 25        |
Detailed analyses of nucleotide databases revealed that this mouse gene maps on chromosome 9 and contains 12 exons spanning more than 21 kb from the start of transcription to the polyadenylation site (data not shown). The predicted mRNA of ~1600 bases in length is translated as a 301-amino acid polypeptide. Analysis of this deduced sequence (accession number NP_076320) predicted a molecular mass of 34 kDa and an acidic isoelectric point (calculated pI of ~4.4), with the presence of an unusual stretch of acidic residues spanning positions 30–52 (Fig. 2). A systematic Blast screening of mouse genomic and expressed sequence tags resources failed to identify paralog genes. However, data base mining indicated the presence of ortholog genes in different species ranging from unicellular eukaryotes (C. reinhardtii and Leishmania major), invertebrates (C. elegans and Drosophila melanogaster), to vertebrates (Danio rerio and Homo sapiens). The alignment of the amino acid sequences predicted for the mouse, human, zebrafish, and worm ORF-2-ortholog genes revealed significant similarity (~70%) throughout the sequence (Fig. 2). The presence of the ORF-2 gene in primitive organisms, such as unicellular flagellates, and the highly conserved nature of the deduced protein sequence throughout evolution, strongly suggest that the corresponding ORF-2 protein is involved in essential cellular mechanisms. Recently two independent groups have characterized the ORF-2 orthologs in C. elegans (19) and C. reinhardtii (20) and reported that these proteins correspond to IFT protein mIFT46. Consequently, we named the mouse ORF-2 gene mIFT46.

**Tissue-specific and Developmental Expression of mIFT46 mRNA**—Gene expression of mIFT46 orthologs has not been analyzed in vertebrates so far. To investigate the tissue distribution of mIFT46 mRNA, total RNAs extracted from different mouse tissues were subjected to Northern blot hybridization. As a positive control, we included total RNA from MC615 chondrocytes. A single transcript of ~1.6 kb was detected at variable levels among the examined tissues, with the strongest levels in ovaries and testis (Fig. 3A). To determine if the activation of mIFT46 gene was linked to development, we examined mIFT46 mRNA expression in total embryos at different developmental stages. Northern blot analysis showed the presence of mIFT46 mRNA as early as stage E8, well before chondrogenesis. Notably, the mIFT46 mRNA level increased at stage E12.5, when skeletogenesis takes place, and remained constant thereafter (Fig. 3B).

**mIFT46 Is Expressed in the Primary Cilium of Chondrocytes**—We generated a polyclonal antibody raised against recombinant mIFT46 to test for existence of endogenous protein, which has never been demonstrated in mammalian cells. This antibody was able to detect the adenovirally overexpressed recombinant IFT46 protein in MC615 cells by Western blotting without any significant cross-reactivity. However, no signal could be seen for the endogenous protein (data not shown), reflecting the very low abundance of this protein. An additional immunoprecipitation step was necessary to enrich the extracts of primary

### FIGURE 1. ORF-2/mIFT46 is a BMP-2-sensitive gene. RT-PCR analysis of ORF-2/mIFT46 expression in MC615 cells (A) and primary embryonic chondrocytes (PEC) (B), in response to BMP-2. MC615 cells and PEC (P0) were grown for 3 days in the presence of 1% FBS alone (0), or supplemented with 50 or 100 ng/ml BMP-2 as indicated, or 10% FBS. Total RNAs extracted from rib cartilage (rbs) of 2-day-old mice were used as a positive control for RT-PCR.

### FIGURE 2. Deduced sequence of the mouse ORF-2/IFT46 protein. Alignment of mouse ORF-2/IFT46 (accession number NP_076320) with human (Q9NQC8), zebrafish (AAI23333), and worm (ABC8650) orthologs. Multialignments were performed with a ClustalW algorithm and ESPript 2.2 software. Identical and similar amino acids are boxed in dark and light gray, respectively.

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It has recently been suggested that IFT46 protein is involved in intraflagellar transport in flagella and non-motile cilia of C. elegans and C. reinhardtii, respectively (19, 20). Because the primary cilium is an organelle present in almost all eukaryotic cells, including chondrocytes (33), we examined the cellular localization of mIFT46 in chondrocytes giving special attention to the cilium. We performed double immunofluorescence analysis on PEC using mIFT46 antibody and an antibody directed against acetylated α-tubulin, a modified isoform of α-tubulin characteristic of microtubules associated with the primary cilium (34, 35). Double staining clearly showed colocalization of mIFT46 and acetylated α-tubulin at the primary cilium of chondrocytes (Fig. 4B). It should be noted that mIFT46 polyclonal antibody reproducibly gave an intense fluorescence signal at the base of the cilium, which becomes more punctuate along it, as illustrated in Fig. 4B. These observations provide the first evidence in favor of the endogenous existence of IFT46 protein in vertebrates and, more precisely, in the primary cilium of mouse chondrocytes.

mIFT46 Is Preferentially Expressed in Differentiated Chondrocytes and/or Early Hypertrophic Chondrocytes in Vitro and in Vivo—We previously reported that BMP-2 can stimulate the differentiated phenotype of chondrocytes and induce their hypertrophic maturation (15, 16). Also, mIFT46 was observed to be a BMP-responsive gene (Fig. 1). We thus examined the expression of mIFT46 mRNA with regard to chondrocyte differentiation status using several in vitro and ex vivo strategies.

As a first in vitro approach, we monitored mIFT46 expression during the de-differentiation process of chondrocytes by using a cellular model we have previously established (17). Freshly isolated embryonic chondrocytes were subcultured as a monolayer on plastic to induce de-differentiation. Our RT-PCR analysis showed that the expression level of the type II procollagen gene (Col2a1), a characteristic marker of differentiated chondrocytes, decreased in P1 chondrocytes subcultured for 3 days in 1% FBS-supplemented medium (Fig. 5A). However, the level of Col2a1 gene expression was sustained with the addition of BMP-2 (Fig. 5A). The transcription factor Sox9 is a major determinant of the chondrogenic lineage during skeletogenesis (36), which is directly involved in the regulation of Col2a1 gene expression (37, 38). In our present cellular system, Sox9 expression was also maintained in presence of BMP-2 (Fig. 5A). Interestingly, mIFT46 expression decreased following chondrocyte de-differentiation in culture but was sustained by BMP-2 treatment (Fig. 5A). Therefore, the mIFT46 gene and the Col2a1 and Sox9 chondrogenic genes responded in a similar fashion to BMP-2 treatment indicating that mIFT46 activation correlates with the differentiation status of the chondrocytes.

We also analyzed mIFT46 expression during the process of chondrocyte maturation. MC615 chondrocytes were cultured with graded concentrations of BMP-2 for 7 days to replicate the progressive switch from a differentiated phenotype toward a mature hypertrophic phenotype. In these culture conditions, Col2a1 expression was stimulated with a concentration of 50 ng/ml BMP-2 but was repressed as the concentrations increased above this level, as previously reported (15). At the same time the expression of the type X procollagen gene (Col10a1), a marker of hypertrophic chondrocytes, was induced with 50 ng/ml BMP-2 and increased in a dose-dependent manner thereafter (Fig. 5B). In this context, the profile of mIFT46 expression showed a pattern similar to that of Col2a1 (Fig. 5B). These observations suggest that mIFT46 expression in chondrocytes is preferentially associated with a differentiated phenotype but not with a mature hypertrophic phenotype.

Subsequently we looked for the localization of mIFT46 expression during endochondral ossification. Cartilage was excised from the anterior part of the rib cage of 17.5-day-old embryos of mouse chondrocytes (PECs) to detect the endogenous protein (Fig. 4A).
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A

B

C

Mouse embryos and was cut in two regions distinguishing non-hypertrophic and hypertrophic chondrocytes. Levels of Col2a1, Col10a1, and mIFT46 gene expression were quantified with real-time RT-PCR. As expected, Col2a1 was mainly expressed in the zone containing non-hypertrophic chondrocytes, whereas Col10a1 was almost exclusively expressed in the hypertrophic zone (Fig. 5C). Even though mIFT46 gene expression was detected in both zones, the steady-state level of mIFT46 mRNA was reduced by \(\sim 2.5\)-fold in the hypertrophic chondrocytes (Fig. 5C). These results confirmed that mIFT46 expression in chondrocytes is preferentially associated with a differentiated state of chondrocytes.

Finally, the patterns of expression of mIFT46, Col2a1, and Col10a1 mRNAs were analyzed and compared by in situ hybridization in distal developing bones of the hind limbs of 17.5-day-old mouse embryos. Strong Col2a1 mRNA signals were detected in all cartilage zones except in hypertrophic chondrocytes and in chondrocytes at the onset of hypertrophy (Fig. 6C). In contrast, Col10a1 showed strong expression in hypertrophic chondrocytes and weak expression in chondrocytes initiating hypertrophic differentiation (Fig. 6E). In parallel tissue sections, mIFT46 showed weak expression in proliferating chondrocytes and in regions corresponding to early hypertrophic chondrocytes, but no signal indicating mIFT46 expression was detected in growth plates containing fully matured hypertrophic chondrocytes (Fig. 6B). In summary, all the different gene expression analyses indicate that mIFT46 expression clearly correlates with a phenotype at a turning point between proliferative and early hypertrophic chondrocytes.

Knockdown of mIFT46 Expression Stimulates Expression of a Subset of Genes in Chondrocytes—As a first attempt to find out if mIFT46 is involved in the regulation of the chondrocyte phenotype, we focused our attention on the loss of function of mIFT46 in cultured chondrocytes using a siRNA approach. Embryonic rib chondrocytes were independently transfected with two different siRNA oligonucleotides targeting mIFT46 or with a siRNA oligonucleotide targeting GFP, as a control. The transfected cells were cultured for 24 h then RNA was extracted. After real-time PCR analysis, we observed a knockdown of mIFT46 expression of \(\sim 80\%\) in chondrocytes upon treatment with each of the two mIFT46 siRNAs (Fig. 7A and data not shown). In these experiments, we analyzed the consequences of mIFT46 gene silencing in chondrocytes by comparing the expression of a focused panel of genes related to skeletogenesis in cells treated or not with mIFT46 siRNA (described under “Experimental Procedures”). We used the commercially available mouse osteogenesis RT² Profiler™ PCR array (Superarray), which makes it possible to follow the expression of 84 genes related to cartilage and bone development, bone mineral metabolism, cell growth and differentiation, extracellular matrix deposition, and cell adhesion. As shown in Fig. 7B, siRNA-mediated mIFT46 silencing selectively stimulated the expression of genes encoding (i) the extracellular matrix proteins biglycan (Bgn) and type XII collagen (Col12a1), (ii) the collagen chaperone Hsp47 (Hsp47), (iii) the matrix metalloproteinase-10 (Mmp-10), (iv) the fibroblast growth factor receptor-1 (Fgfr1), and (v) the transcription fac-

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**FIGURE 5.** Expression of mIFT46 mRNA is associated with a differentiated phenotype and/or early hypertrophic chondrocyte phenotype. A, mIFT46 expression correlates with the state of chondrocyte differentiation. Chondrocytes were extracted from rib cages of mouse embryos and cultured for 1 week (P0) in presence of 10% FBS, then were trypsinized and subcultured for 3 days (P1) in the presence of 1% FBS alone or supplemented with 50 ng/ml BMP-2, as indicated. Total RNAs were extracted at the indicated times and were submitted to RT-PCR analysis. Note that BMP-2 sustains expression of mIFT46, Col2a1, and Sox9, when compared with 1% FBS alone. Total RNAs extracted from freshly isolated chondrocytes (FC) were used to evaluate the initial level of gene expression. B, dose-dependent effect of BMP-2 on mIFT46 expression. MC615 cells were grown for 7 days in the presence of 1% FBS alone (0) or supplemented with 50–400 ng/ml BMP-2 as indicated, and the expression of mIFT46, Col2a1, and Col10a1 was analyzed by RT-PCR. Note the parallel responsiveness of mIFT46 and Col2a1 gene expression to BMP-2 and the highest level of Col10a1 expression concomitant with the lowest level of Col10a1 expression. C, quantitative RT-PCR analysis of expression of mIFT46, Col2a1, and Col10a1 in non-hypertrophic (Ch.) and hypertrophic chondrocytes (Hyp. Ch.) isolated from rib cages of 17.5-day-old mouse embryos.
tor Msx1 (Msx1). The expression of the other genes involved in chondrogenesis (including Col2a1, Sox9, and Col10a1) were not significantly modulated by mIFT46 knockdown. This PCR array analysis thus revealed that knockdown of mIFT46 leads to the activation of a subset of genes in chondrocytes.

Expression of zIFT46 during Zebrafish Development—Northern blotting analysis showed that mIFT46 is expressed well before chondrogenesis during mouse embryonic development (Fig. 3B). Considering the IFT character of IFT46, this result is consistent with the known existence of primary cilium in most embryonic and adult cells. Therefore, we decided to investigate the role of IFT46 during early development. Among the different vertebrate models allowing the study of protein function in early development, the zebrafish offers a number of advantages, such as rapid development, embryo transparency, and genetic accessibility. We first examined the zIFT46 expression pattern during zebrafish early development by real-time RT-PCR and whole mount in situ hybridization (Fig. 8). As illustrated in Fig. 8A zIFT46 was found ubiquitously but weakly expressed at different time points during the first steps of development. More precisely, the presence of zIFT46 transcripts was initially detected at 3 h post fertilization (hpf) indicating the maternal origin of this mRNA (Fig. 8). Then the zIFT46 gene is activated in the embryo as early as the beginning of the epiboly movement (5 hpf) and later during development (Fig. 8, A and B). A peak of expression was noted at 20 hpf, which corresponds to late somitogenesis and establishment of the first cartilage elements (Fig. 8B).

Knockdown of zIFT46 Affects Zebrafish Development—The role of zIFT46 during early development was further analyzed by injecting an antisense oligonucleotide (morpholin or (MO)) designed to specifically knockdown the expression of zIFT46 at the translational level. Fluorescein isothiocyanate-labeled morpholinos were injected at the 1–4 cell stage: only embryos showing homogeneous distribution of fluorescein isothiocyanate at 5 hpf were selected for subsequent observations. An antisense morpholin termed zIFT46-MO, hybridizing at the vicinity of the start codon, was used for zIFT46 knockdown. In parallel, a morpholin containing five mismatches, termed 5mis-MO, was used as a control. The embryos injected with 5mis-MO behaved like non-injected embryos. In contrast, most embryos injected with zIFT46-MO (designed as morphants) exhibited morphological defects during development (Fig. 9, A–D, and see Table 2). During gastrulation the shape of the embryos became ovoid rather than round (Fig. 9A). Then dur-
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During somitogenesis, the tail bud extended off the yolk prematurely and appeared shortened (Fig. 9B). At 24 hpf, most morphants (~80%) showed a body axis wound up in a snail shell-like fashion (Fig. 9C). All together these observations indicated that morphants presented clear signs of dorsalization, which might be a consequence of ventral mesoderm limitation and/or dorsal mesoderm expansion. The broad spectrum of these dorsalization phenotypes, examined at 24 and 48 hpf, could be scored in three categories (C1–C3) (Fig. 9D and Table 2). Interestingly, a small proportion of the 24-hpf morphants (~6%) presented a nascent ectopic tail (Fig. 9C and Table 2). Phenotype specificity of the morphants was confirmed by coinjecting the in vitro transcribed zIFT46 mRNA together with zIFT46-MO. In these conditions the phenotype of the injected embryos was normal (Fig. 9C and see Table 2). We also checked that the injection of zIFT46 mRNA alone resulted in normal embryo development (Fig. 9C).

We then analyzed the phenotypes of the different morphants at the molecular level by using in situ hybridization. In a previous study in zebrafish, Mullins et al. (39) detailed a series of dorsalization phenotypes of varied strengths generated by ethynitrosourea mutagenesis. The mutant embryos presented an ovoid shape with a widening of the notochord at the tail bud stage and, later during development, a tail wound-up on the back (39). Thus to better characterize the morphological and structural changes occurring in the zIFT46 morphants, we used in situ hybridization to analyze the expression of the zebrafish Col2a1 gene (zCol2a1), a characteristic marker of the differentiating notochord (40). At 13 hpf, the zCol2a1 hybridization signal clearly reveals enlargement of the notochord in zIFT46-MO-injected embryos compared with wild-type (Fig. 10A). We also examined more closely the morphants showing an ectopic tail at 24 hpf to evaluate the molecular nature of this supernumerary element. The zCol2a1 transcripts were detected both in morphants and wild-type embryos in the floor plate and hypochord in the trunk, and in the notochord at the tip of the primary tail (Fig. 10B). In addition, zCol2a1 was clearly expressed in the ectopic tail of the morphants (Fig. 10B), indicating the presence of the same cell types as in the primary tail, that is notochord and/or hypochord cells. To determine whether the ectopic tail, like the primary tail, is induced by a tail-organizing center, we examined the localization of eve1 expression by in situ hybridization. eve1 is a zebrafish homeobox gene expressed in the ventral mesoderm during gastrulation and in the tail.

FIGURE 8. zIFT46 expression in the zebrafish embryo. A, whole mount in situ hybridization showing ubiquitous and weak expression of zIFT46 during embryogenesis. Panels of embryos at 3, 5, and 12 hpf are viewed from the side with the animal pole placed on the top. B, quantitative RT-PCR analysis of zIFT46 expression at the indicated developmental stages. Histograms represent the ratio of expression between zIFT46 and the housekeeping gene zhistone2A.

FIGURE 9. Knockdown of zIFT46 expression in zebrafish embryos induces a dorsalization phenotype or the formation of an ectopic tail. A, ventral views of 5mis-MO- and zIFT46-MO-injected embryos at 11 hpf. The animal pole is up. Note the ovoid shape of the morphant. B, lateral views of wild-type (WT) and zIFT46-MO-injected embryos at 14 hpf, observed with bright light (on the left) or fluorescence (on the right). Note that the tail of the morphant extends from the yolk. H, head; T, tail. C, lateral views of wild-type (WT), zIFT46-MO, zIFT46 mRNA-injected, and zIFT46-MO/zIFT46 mRNA co-injected embryos at 24 hpf. Two types of morphological abnormalities are displayed by the morphants, which are (i) a caudal segment wound up in a snail shell-like fashion (asterisk) or (ii) an ectopic tail (arrowhead). Note the fully formed tail in embryos injected with zIFT46 mRNA alone or coinjected with zIFT46 mRNA together with zIFT46-MO. In this last case observation with fluorescence shows that, despite the distribution of zIFT46-MO in the whole embryo, injection of zIFT46 mRNA rescues the phenotype. D, lateral views of wild-type (WT) and zIFT46-MO embryos at 48 hpf, ranged in three phenotypic classes from the less (C1) to the most severe dorsalization (C3) phenotype. C1 corresponds to a tail bent upward. C2 corresponds to a wound-up tail closely resembling to a piggy tail. C3 corresponds to a shortened and twisted tail.
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**TABLE 2**
Effect of morpholinos on development of zebrafish embryos at 24 hpf

| Injections               | Number of embryos | WT phenotype | Dorsalized phenotype | Ectopic tail |
|--------------------------|-------------------|--------------|----------------------|--------------|
|                          | n     | %     | C1 + C2 + C3          |              |
| zIFT46-MO                | 128   | 13    | 81                    | 6            |
| 5mis-MO                  | 107   | 98    | 2                     | 0            |
| zIFT46-MO + zIFT46 mRNA  | 45    | 100   | 0                     | 0            |
| Phenol red               | 77    | 99    | 1                     | 0            |
| Non-injected             | 240   | 100   | 0                     | 0            |

The injections were performed at the 1–4 cell stage. Concentrations of injected morpholinos were 1 mM. *In vitro* transcribed zIFT46 mRNA was used at 100 ng/μl. Phenol red was used as mock injection.

**DISCUSSION**

**mIFT46 Is Localized in the Primary Cilium in Chondrocytes—**
In the present study we cloned *mIFT46* as a BMP-2-responsive gene whose product is localized in the primary cilium in mouse chondrocytes. Previous reports have already shown that chondrocytes exhibit a primary cilium, which projects into the extracellular matrix to interact with molecules such as collagen and proteoglycans via several receptors, including integrins (34, 43–46). The function of such a microtubule-based organelle is currently unknown in chondrocytes. Poole and colleagues (34) proposed that primary cilia in chondrocytes play the role of mechanical sensors. This function has already been described in other tissues such as kidney epithelium (47), but there is currently no evidence to support this hypothesis in cartilage.

Time lapse images of the movement of mIFT46-GFP within the ciliated endings of neurons in *C. elegans* suggested that IFT46 (designated as Dyf-6) is involved in IFT (19). Very recently a study suggesting the localization of *mIFT46* in the primary cilium of mouse chondrocytes by using an antibody against mIFT46 (Fig. 4). It is known that proteins required for IFT concentrate at the base of cilia, where they assemble into large protein complexes called IFT particles first described in *Chlamydomonas* (48, 49). The IFT particles are trafficked along the axoneme by heterotrimeric kinesin-II and a cytoplasmic dynein in the anterograde and retrograde directions, respectively. The subcellular localization of mIFT46, visualized for the first time at physiological levels (Fig. 4), strongly suggests that mIFT46 is indeed involved in IFT. In particular, the punctate fluorescence present along the cilium, corresponding to mIFT46, probably indicates the different positions of mIFT46 during the transport process. In addition, mIFT46 is more intensely stained at the base of cilia, in the basal body, suggesting its transient storage in this zone to enable it to participate in intraflagellar transport. This particular localization of mIFT46 might require the participation of its N-terminal extremity. Indeed, acidic clusters are found in a variety of cilia proteins such as nephrocystin. Nephrocystin interacts with the transport protein phosphofurin acidic cluster sorting protein-1 via its acidic cluster, which is essential to target the protein at the base of the cilium (50).

We also described that *mIFT46* expression occurs early in embryonic development, before chondrogenesis, and is sustained in a variety of adult tissues. This suggests a diversity in the function of *mIFT46*. In agreement with this view, cilia with IFT particles are expressed on most embryonic and adult cells, indicating indeed that IFT proteins/cilia may have different functions in specific cell types or during distinct stages of development. This is particularly well illustrated by the loss of the IFT protein polaris in mice resulting in a complex series of pathologies involving not only the kidney, liver, brain, testis, eye, pancreas, and skeleton (51–54) but also left-right axis determination in the embryo (55).

**FIGURE 10.** Molecular characterization of *zIFT46-MO* phenotypes by *in situ* hybridization. *A*, dorsal views of embryos at 13 hpf, showing *zCol2a1* expression in the notochord. The animal pole is up and the notochord is delineated by *arrowheads*. Note the broader and less defined staining of *zCol2a1* in the *zIFT46-MO*-injected embryo. Note also that the morphant appears more elongated than the wild-type embryo. *B*, *top*, lateral views of wild-type and *zIFT46-MO* embryos at 24 hpf, showing *zCol2a1* expression in the trunk (floor plate (*fp*) and hypochord (*hy*)) and in the notochord (*no*) at the tip of the primary tail. Note the presence of *zCol2a1* expression in the ectopic tail of *zIFT46-MO* embryo. *Bottom*, higher magnification of the primary and secondary tails at 24 hpf, in wild-type or *zIFT46-MO*-injected embryo. *Inset* shows *eve1* expression at 22 hpf (at the onset of ectopic tail appearance) at the tip and in the ventral axis of the tail of *zIFT46-MO*-injected embryo. *Arrowheads* indicate the location of the ectopic tail in each panel.
expression of mIFT46 and Col2a1 in chondrocytes was observed in response to BMP-2. In addition, the opposite response obtained by increasing the dose of BMP-2 observed upon mIFT46 and Col10a1 expression reveals that mIFT46 expression in chondrocytes is closely associated with the differentiated phenotype and/or early hypertrophic phenotype, but not with the late hypertrophic phenotype. This is in accord with the results of our RT-PCR analysis of rib cartilage showing preferential expression of mIFT46 in the zone containing small proliferative chondrocytes. The expression of mIFT46 found in the zone of hypertrophic chondrocytes is likely due to the additional presence of early hypertrophic chondrocytes, because no intermediate region between proliferative and hypertrophic zone was removed during the dissection. At any rate, the in vivo correlation of mIFT46 expression with the expression of a phenotype characteristic of differentiated and/or early hypertrophic chondrocytes was unequivocally demonstrated by our in situ hybridization analysis revealing mIFT46 expression in cartilage in regions associated with the onset of chondrocyte hypertrophic differentiation. At E17.5, in situ hybridization analysis indicated that mIFT46 is expressed in the prospective growth plates of the future ossification center in metatarsus, but not in the well formed growth plate in the central core of tibia where chondrocytes appeared largely hypertrophic. This difference could conceivably be related to the different stages of growth plate development in metatarsus and tibia. Additionally, it could also be related to the different states of cell differentiation from the periphery to the center of the tibial growth plate. Examination of cross-sections of cartilage rudiments in the developing limb bud have shown that gene or protein expression of type II collagen and cartilage proteoglycan aggregate can be consistently stronger in the peripheral zone compared with the center; this is indeed related to the proliferation, maturation, or hypertrophy of the chondrocytes in the different areas of the rudiments (56, 57).

**Knockdown of mIFT46 Stimulates Selective Gene Expression in Chondrocytes**—An increasing number of studies suggests that primary cilia/IFT proteins are essential components of morphogenetic signaling pathways for skeletal development (53, 54, 58–61). In developing long bones it is known that Indian hedgehog (Ihh) and the parathyroid hormone-related peptide act together to regulate the onset of chondrocyte hypertrophy in a negative feedback loop (reviewed in Ref. 62). In this regard, it is interesting to note that vertebrate Smooth-end (Smo), the Hedgehog (Hh) signaling receptor, functions at the primary cilium (63). However, if the requirement of the cilium to transmit Hh signaling has been well established, a very recent study with the conditional allele of the IFT protein Ift88/tg737 (polaris) has demonstrated that the role of IFT and cilia during endochondral bone development is not restricted in Hh signaling and may include a role for cilia or IFT in additional signaling pathways such as the Wnt pathway (58). In the growth plate important signaling molecules such as Wnt, FGF, and BMP are known to be involved in the regulation of chondrocyte differentiation (64–68). In the study presented here we show that mIFT46 is a BMP-2-responsive gene, suggesting therefore that BMP signaling could modulate the functionality of the primary cilium in chondrocytes.

As a first attempt to find out how mIFT46 could be involved in the regulation of the chondrocyte phenotype, we analyzed the consequence of mIFT46 gene silencing in chondrocytes by focusing our attention on a panel of genes related to skeletogenesis. Knockdown of mIFT46 in chondrocytes did not affect the expression of the major genes signaling the differentiated phenotype (such as Col2a1) or the hypertrophic phenotype (such as Col10a1), nor the expression of genes coding for important BMP signaling molecules (such as BMP receptors or Smads) (data not shown). However, knockdown of mIFT46 in chondrocytes led to the up-regulation of several genes: Msx1, Fgfr1, Col12a1, Bgn, Mmp10, and Hsp47. Msx1 is a transcription factor particularly important for craniofacial development (69), but its role in chondrocyte differentiation is still not clear (70). Fgfr1 codes for a FGF receptor that transmits a mitogenic signal in chondrocytes both in vitro and in vivo. Fgfr1 is expressed in pre-hypertrophic and hypertrophic chondrocytes in the growth plate (71, 72). Moreover, BMP and FGF signaling have opposite actions in the growth plate (65), and recent work suggests that BMP pathways antagonize FGF pathways in part by inhibiting expression of FGF1 (68). Whether or not IFT46 is involved in this balance between BMP signaling and FGF signaling remains to be determined, but it is interesting to note that FGF1 is associated with cilia in cells of the tracheal ring (73), suggesting a close relationship between cilia and FGF signaling. The Col12a1 and Bgn gene products are components of the macromolecular network of cartilage matrix (74, 75). In particular, type XII collagen is found in the septa of stacked chondrocytes forming the growth plate (74). Biglycan is usually associated with cartilage matrix mineralization and is prominently expressed by hypertrophic chondrocytes (76). The collagen chaperone Hsp47 is present in the hypertrophic region of cartilage (77, 78). Matrix metalloproteinase 10 (MMP-10) promotes collagenolysis via activation of other MMPs, which play an important role in cartilage degradation. MMP-10 is also expressed by hypertrophic chondrocytes (79, 80). Thus the genes that were up-regulated by mIFT46 knockdown are involved in growth plate physiology. Taken together the results presented here suggest that mIFT46 could play a role at the onset of chondrocyte hypertrophy. In the same vein, McGlashan et al. (54) have shown very recently that loss of the IFT protein solaris and failure to form a functional primary cilium in mice affect chondrocyte differentiation and result in delayed chondrocyte hypertrophy within the growth plate. In another recent study, Kif3a, a subunit of the Kinesin-II motor complex required for IFT and the formation of cilia, was deleted in mouse chondrocytes (81). In this latter study, deletion of IFT and cilia resulted in post-natal dwarfism due to premature loss of growth plate, with alterations in proliferation, differentiation, cell shape, and rotation of the chondrocytes (81). Thus increasing evidence suggests that IFT/cilia are required for proper differentiation and maturation of chondrocytes and growth plate development. Clearly further studies using conditional knockout mice are needed to determine precisely the role of the ciliary protein IFT46 in chondrocytes and in endochondral ossification.

**Knockdown of zIFT46 Affects Early Development in Zebrafish**—We also explored the role of IFT46 during early development...
using the zebrafish model. We first examined *zIFT46* expression during development of the zebrafish by *in toto* hybridization. At all time points examined during the first 24 h of development, *zIFT46* transcripts were weakly detected in all regions of the embryos. Only a few IFT genes have been examined in zebrafish, and the developmental study of one of them, *hi409/IFT81*, reveals also wide expression during early development (82). Furthermore, our quantitative RT-PCR and *in situ* hybridization analyses indicated that *zIFT46* is expressed in zebrafish embryos as a maternal transcript. This is in complete accordance with the maternal contribution reported for other IFT genes in zebrafish (82). This maternal contribution suggests that *zIFT46* is required very early in development and in the formation and/or function of cilia. Indeed, in addition to their roles in signaling pathways, it is known that cilia play critical roles in early patterning of the body plan of vertebrate embryos. For instance, the cilia on the ventral node of vertebrate embryos are required for the normal establishment of left–right asymmetry (83).

Our examination of the zebrafish genome draft provided no evidence for *IFT46* gene duplication. Moreover, the IFT46 protein sequence does not share any sequence similarity with other proteins. This information encouraged us to carry out loss-of-function experiments to investigate the endogenous role of *zIFT46* during early development of zebrafish, because genetic functional redundancy can be excluded *a priori*.

Injection of an antisense *zIFT46* morpholino oligonucleotide in zebrafish embryos dorsalized most embryos. It should be noted that no sign of left-right asymmetry was observed. Early dorso-ventral pattern formation in vertebrate embryos is regulated by various factors such as BMPs and Wnts, and their antagonists (84). During gastrulation in zebrafish, BMP signaling is activated to a greater degree on the ventral side of the embryo where it patterns the mesoderm to adopt different fates, including blood, vasculature, pronephros, and tail muscle. It is also well known that loss of BMP signaling in zebrafish leads to expansion of dorsal structures at the expense of ventral structures (85, 86). Interestingly the dorsalization phenotypes displayed by the *zIFT46* morphants phenocopy the phenotypes described for a number of zebrafish mutants of the BMP pathway. For instance, we have observed that the shape of most *zIFT46*-MO embryos becomes ovoid at the bud stage, also the notochord is broader and the tail bud extends off the yolk prematurely, then the embryos develop with a shortened and/or twisted tail. These morphological defects are visible in zebrafish mutants lacking BMP-2B, BMP-7, Alk-8, or Smad5 (85–88). Moreover, a minority of *IFT46*-MO embryos developed an ectopic tail. Interestingly, Pyati et al. (89) have shown that after early gastrulation BMP signaling plays a role in preventing the formation of ectopic tails in zebrafish. Altogether the similarities between the morphological defects displayed by the *zIFT46* morphants and the phenotypes characteristic of the mutants of the BMP signaling pathway suggest that *zIFT46* is involved in BMP signaling pathway. Our finding that *IFT46* is a BMP-2-sensitive gene supports this view, but other studies at the molecular level are strongly needed to determine the role of *IFT46* protein with respect of BMP signaling.

**Concluding Remarks**—Our results show for the first time the endogenous protein IFT46 is found in the primary cilium of vertebrate cells. Its punctate localization along the cilium strongly suggests its involvement in IFT. Importantly, our results indicate that IFT46 may play a role in chondrocyte maturation and in early development. Very recently the first chondrodysplasia to be linked to a defect in IFT or primary cilium function has been described (90). Mutations in IFT80 provoke Jeune asphyxiating thoracic dystrophy in young individuals, characterized by skeletal manifestations only, whereas knockdown of IFT80 in zebrafish result in several defects, including a large kidney cyst, severe cardiac edema, and a curled tail (90). Therefore, the study presented here adds to the increasing body of evidence suggesting that IFT proteins can play diverse roles in development and organogenesis, including skeletogenesis. Now it will be of particular interest to investigate how IFT46 integrates into signaling pathways to coordinate a specific cellular response.

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