Ciliary IFT80 balances canonical versus non-canonical hedgehog signalling for osteoblast differentiation

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Intraflagellar transport proteins (IFT) are required for hedgehog (Hh) signalling transduction that is essential for bone development, however, how IFT proteins regulate Hh signalling in osteoblasts (OBs) remains unclear. Here we show that deletion of ciliary IFT80 in OB precursor cells (OPC) in mice results in growth retardation and markedly decreased bone mass with impaired OB differentiation. Loss of IFT80 blocks canonical Hh–Gli signalling via disrupting Smo ciliary localization, but elevates non-canonical Hh–Gαi–RhoA–stress fibre signalling by increasing Smo and Gαi binding. Inhibition of RhoA and ROCK activity partially restores osteogenic differentiation of IFT80-deficient OPCs by inhibiting non-canonical Hh–RhoA–Cofilin/MLC2 signalling. Cytochalasin D, an actin destabilizer, dramatically restores OB differentiation of IFT80-deficient OPCs by disrupting actin stress fibres and promoting cilia formation and Hh–Gli signalling. These findings reveal that IFT80 is required for OB differentiation by balancing between canonical Hh–Gli and non-canonical Hh–Gαi–RhoA pathways and highlight IFT80 as a therapeutic target for craniofacial and skeletal abnormalities.
Primary cilia are highly conserved microtubule-based organelles that project from the cell surface into the extracellular environment and play essential roles in vertebrate development, cellular differentiation, proliferation, sensory transduction and homeostasis. Cilia formation and function requires effective intraflagellar transport (IFT), a bidirectional driving system run by IFT protein complexes and two kinds of motors (kinesin and dynein). Primary cilia and IFT proteins are present on almost all vertebrate cells including osteoblasts (OBs). Growing evidence has shown that primary cilia and IFT proteins are integral to the aetiology of skeletal and craniofacial disorders, owing to their role in bone related signal transduction, such as hedgehog (Hh) signalling. Other studies demonstrated that IFT proteins are also involved in the regulation of bone homeostasis through mechanosensation. However, the mechanism of those proteins in regulating OB differentiation and function during skeletal development remains largely unknown.

Emerging evidence demonstrated that the mutations of cilia-related proteins such as polycystin-1 (Pkd1, a large transmembrane-spanning glycoprotein) and Ki3a (a member of kinesin family) in OPGs result in impaired OB differentiation and osteopenia. Further studies have shown that conditional deletion of Pkd1 or Ki3f in mature OBs impairs OB sensory function and disrupts mechanosensation-mediated skeletal homeostasis. Despite the new insights on these ciliary proteins, the underlying molecular mechanisms of IFT proteins remain unclear.

Recent studies have shown that mutations of IFT and other cilia-related proteins significantly affect Hh canonical signalling, but display a variety of phenotypes, indicating that individual IFT protein plays different roles in the regulation of Hh signalling. Primary cilia provides a specialized environment hosting the key components of the Hh canonical signal pathway, including Hh receptor Patched 1 (Ptc1), Smoothened (Smo) protein—a G protein coupled receptor (GPCR) and Gli transcription factors. When Hh is present and bounds to Ptc1, Ptc1 releases its inhibition on Smo and allows Smo to translocate into cilia. In cilia, active Smo causes the dissociation of Gli from Suppressor of Fused (SUFU), which allows Gli to be processed into its activated form. In OBs, activated Gli regulates BMP2 and Runx2 expression, which are critical regulators of OB differentiation and bone formation. Therefore, Hh–Gli signalling plays the critical role in OB differentiation and bone development. Mice lacking Shh fail to form vertebrae and limbs due to the inhibition of endochondral ossification, confirming that Shh is indispensable for bone formation. However, the most recent evidence has shown that not all Hh signalling goes through Gli activation, the canonical Hh signalling pathway. Gli-independent Hh signalling is recognized and referred to as the non-canonical Hh signalling. One of the non-canonical Hh signalling activates small GTPases RhoA and Rac1 via Smo-coupled Gz branches and regulates actin cytoskeleton in fibroblasts and endothelial cells. Nevertheless, whether and how non-canonical Hh signalling regulates OB differentiation is unknown.

IFT80 is an IFT protein in IFT complex B. Mutations with reduced expression of IFT80 in human cause Jeune asphyxiating thoracic dystrophy (JATD) and short rib polydactyly type III (SRP III). Both diseases have bone abnormalities including shortening of the long bones and constriction of the thoracic cage. JATD and SRP III often lead to death in the prenatal stage or during infancy due to respiratory insufficiency. However, it is still unknown about the role and mechanism of IFT80 in bone development and OB differentiation. Previously, we found that IFT80 regulates osteogenesis through Hh–Gli signalling in vitro. In this study, by using IFT80 conditional knockout model, we further found that IFT80 is essential for balancing canonical and non-canonical Hh signalling pathways in OB differentiation. Deletion of IFT80 disrupts canonical Hh–Gli activation but overactivates non-canonical Hh–Gz–RhoA–ROCK (Rho-associated coiled-coil-containing kinases) pathway through altering Smo ciliary location and increasing Gz and Smo binding. Inhibition of RhoA or ROCK promotes ciliogenesis and treatment of IFT80-deficient OPCs with cytchalasin D (Cyto D), a filamentous actin (F-actin) destabilizer, greatly restores OB differentiation through promoting canonical Hh–Gli transduction and inhibiting Hh–RhoA–stress fibre non-canonical signalling. These findings reveal dual regulation of IFT80 in Hh signalling. On one hand, IFT80 promotes Hh canonical signalling via activation of Hh–Smo–Ptc1–Gli signalling pathway; on the other hand, IFT80 inhibits Hh non-canonical signalling via Hh–Gz–RhoA–stress fibre signalling in OB differentiation.

**Results**

**IFT80 deletion causes growth retardation and osteopenia.** The mouse IFT80 gene spans 142,546 bp of genomic DNA and contains 19 exons on chromosome 3. We generated IFT80−/− mice that contain two loxP sites flanking exon 6, as described in Methods (Supplementary Fig. 1a). Homozygous IFT80−/− mice exhibited normal lifespan and fertility without any apparent phenotypic abnormality. Genomic DNA from tails was used for PCR genotyping. The 469-bp band corresponds to IFT80−/− mice and the 247-bp band corresponds to wild-type (IFT80+/+) (Supplementary Fig. 1b). To understand the role of IFT80 in bone development, we generated OSX-Cre;IFT80−/− mice (referred to as OSX;IFT80−/−) by crossing IFT80−/− mice with OSX-Cre transgenic mice. Western blot analysis confirmed that the expression of IFT80 was significantly decreased in the calvarial bone of OSX;IFT80−/− mice (Supplementary Fig. 1c). Quantitative PCR (QPCR) analysis showed that IFT80 expression was almost absent from OPCs derived from the calvarial bone of OSX;IFT80−/− mice (Supplementary Fig. 1d). OSX;IFT80−/− mice were born with expected Mendelian ratios, but showed growth retardation (Supplementary Fig. 2a) accompanied with significantly decreased body weight starting at postnatal day 5 (Supplementary Fig. 2b). In addition, OSX;IFT80−/− mice showed shorter body length starting from 1 month old (Supplementary Fig. 2c). Tibiae length of newborn OSX;IFT80−/− mice were slightly shorter than that of OSX;IFT80+/+ mice, however, tibiae length of 4-month old OSX;IFT80−/− mice were significantly shorter than that of OSX;IFT80+/+ mice (Supplementary Fig. 2d). Microcomputed tomography (Micro-CT) results of femurs from one-month-old mice showed an apparent decrease in the bone mass of OSX;IFT80−/− mice in both trabecular and cortical bone with apparent bone defects (Fig. 1a). The percentage of bone volume to total bone volume (BV/TV), trabecular thickness (Tb.Th) and trabecular number (Tb.N) of OSX;IFT80−/− mice were, respectively, 0.34, 0.35 and 0.55-fold of that in OSX;IFT80+/+ mice (Fig. 1b). Trabecular spacing (Tb.Sp) of OSX;IFT80−/− mice was almost twofold of that in OSX;IFT80+/+ mice (Fig. 1b). These data suggest an osteopenia phenotype. Moreover, OSX;IFT80−/− mice also displayed narrow rib cages (Supplementary Fig. 2e) that mimic the patients with IFT80 mutations.

To further confirm the phenotype and fully understand the role of IFT80 in skeletal development, histological haematoxylin and eosin (H&E) staining analysis was performed in the tibiae section of OSX;IFT80−/− mice. The tibiae from newborn and 1-month-old OSX;IFT80−/− mice all showed decreased trabecular bone...
Deletion of IFT80 blocks OB differentiation. To determine whether the decreased bone mass phenotype of IFT80f/f mice resulted from impaired OBs, we studied the role of IFT80 in OB differentiation, function and proliferation in vitro. Primary OPCs were isolated from IFT80+/+ mice, and infected with Ad-CMV-Cre to delete IFT80 as described in Methods (referred to as IFT80/d). Ad–green fluorescent protein (Ad–GFP)-infected IFT80+/+ cells were used as control and still marked as IFT80+/+. Ad–GFP infection showed similar osteogenesis as Ad–CMV-Cre infection in wild-type OBs as detected by ALP activity and Alizarin Red staining (Fig. 2a,b), suggesting Ad-CMV-Cre has no significant effect on OB differentiation compared with Ad–GFP. Ad–GFP-infected IFT80+/+ cells showed higher GFP signalling in the beginning of osteogenesis but reduced at day 14 of OS induction (Supplementary Fig. 3a,b). Western blot and QPCR showed that Ad-CMV-Cre transduction yielded 95% reduction of IFT80 in IFT80+/+ OPCs (Fig. 2c,d). Deletion of IFT80 in OPCs impaired OB differentiation and maturation as shown with less ALP activity (Fig. 2e), reduced Alizarin Red accumulation (Fig. 2f) and lower expression level of OB differentiation markers, including Runx2, osterix and osteocalcin (Fig. 2g). Compared with that in IFT80+/+ OBs, phosphorylation of Smads 1/5/8 in IFT80/d OBs was significantly reduced (Fig. 2h). These data are consistent with the bone defect phenotypes observed in IFT80+/+ mice and highlight the important role of IFT80 in OB differentiation. However, we cannot simply rule out the role of IFT80 in OB proliferation. Ki67 staining and MTS assay were performed to study cell proliferation and the data showed that deletion of IFT80 also inhibited the OB proliferation (Supplementary Fig. 4a,b). Hh agonist purmorphamine did not promote cell proliferation in both IFT80+/+ and IFT80/d groups (Supplementary Fig. 4c). To eliminate the proliferation effect on cell differentiation, we performed all the differentiation-related assays under the condition that the cells have reached confluency as both IFT80+/+ and IFT80/d showed similar and low-proliferation effect (Supplementary Fig. 4d).

IFT80 deletion disrupts ciliogenesis and Hh–Gli signalling. To further confirm that IFT80 was deleted after Ad-CMV-Cre infection, we performed immunofluorescence staining for IFT80 in OPCs from OSX;IFT80+/+ and OSX;IFT80+/+ mice. In
OSX;IFT80+/+ OPCs, IFT80 protein was detected in the basal bodies and along the ciliary axoneme. However, IFT80 was absent in OSX;IFT80f/f OPCs (Fig. 3a). To further analyse the role of IFT80 in ciliogenesis, we performed double staining of the basal body (anti-γ tubulin) and axoneme (anti-acetylated α-tubulin) to visualize cilia. About 80% of the cells in OSX;IFT80+/+ group have normal cilia structure, while there were substantially fewer ciliated cells in OSX;IFT80f/f group. More than 60% of the cells lost cilia structure and only the centrosomes were stained in OSX;IFT80f/f group. About 30% of the cells had cilia structure but displayed shortened cilia compared with OSX;IFT80+/+ group (Fig. 3b). These data indicate that IFT80 is essential for cilia formation.

To explore the mechanisms by which loss of IFT80 blocks OB differentiation, we characterized the effect of IFT80 deletion on Hh–Gli canonical signalling pathway. Treatment of IFT80f/f OPCs with Shh (1 μg ml⁻¹) resulted in a significant promotion in OB differentiation (Fig. 3c,d) and a significant increase in the expression of Gli1 and Ptc1h, indicating the activation of Hh signalling (Fig. 3e). In contrast, Shh failed to similarly stimulate OB differentiation, elevate Gli1 and Ptc1h expression (Fig. 3c–e), and promote Gli-responsive-promoter luciferase activity in IFT80f/−/f OPCs (Fig. 3f). Hh agonist purmorphamine also failed to stimulate OB differentiation in IFT80f/−/f group (Supplementary Fig. 5a,b). These data suggest that loss of IFT80 impairs Hh–Gli signalling in OPCs. Consistent with previous reports that Hh signalling enhances Runx2 and BMP2 expression,20,21 we found that Shh promoted Runx2 and BMP2 expression in IFT80f/f OPCs (Fig. 3g) but failed to increase the expression of these genes in IFT80f/−/f OPCs (Fig. 3g). These results demonstrate that deletion of IFT80 blocks OB differentiation due to impaired cilia formation and Hh–Gli transduction.

Since BMP2 and Runx2 act downstream of Hh–Gli signalling, we tested whether overexpression of BMP2 or Runx2 could restore osteogenesis in IFT80d/d OPCs. Infection of IFT80f/f and IFT80f/−/f OPCs with Ad-BMP2 or Ad-Runx2 significantly promoted BMP2 or Runx2 expression (Supplementary Fig. 6a,b). Overexpressing either BMP2 or Runx2 partially rescued osteogenesis in IFT80f/−/f OPCs, as evidenced by restored ALP activity (Fig. 3h), Alizarin Red accumulation (Fig. 3i), and the expression of osterix and osteocalcin (Fig. 3j). However, overexpression of BMP2 or Runx2 could not fully restore IFT80f/−/f OB differentiation, suggesting that a parallel mechanism might exist to cooperate with Hh–Gli pathway to regulate OB differentiation.

**IFT80 deletion activates non-canonical Glii–RhoA signalling.** Given that cilia are associated with actin cytoskeleton,36 we analysed actin stress fibre density and found that deletion of IFT80 significantly increased stress fibre density (Fig. 4a,b). RhoA is a small GTPase protein, which regulates actin cytoskeleton.37 Therefore, we measured RhoA activity during OB differentiation. Consistent with the stress fibre results, RhoA activity was significantly reduced along the differentiated progression of both IFT80f/f and IFT80f/−/f OBs (Fig. 4c). However, IFT80f/−/f OBs showed significantly higher RhoA activity than IFT80f/f OBs during OB differentiation (Fig. 4c).

Non-canonical Hh signalling pathway has been reported in endothelial cells, which regulates actin stress fibres in response to Hh via a Gli-independent mechanism.30 Since Hh–Gli transduction was blocked and RhoA activity and stress fibre were increased in IFT80f/−/f OPCs, we hypothesized that IFT80 regulates RhoA–stress fibre signalling in a Hh-dependent manner and that loss of IFT80 shifts activation of Hh signalling from the...
Figure 3 | Deletion of IFT80 disrupts cilia formation and inhibits canonical Hh–Gli signalling transduction in OPCs. (a) Immunofluorescence analysis of IFT80 in IFT80f/f and IFT80d/d OPCs. IFT80 (red) was co-stained with acetylated tubulin (ciliary axoneme, cyan). DAPI (nuclear marker) staining is used as counterstaining. Scale bars, 10 μm. (b) Immunofluorescence analysis of primary cilia in IFT80f/f and IFT80d/d OPCs. Primary cilia were stained with γ-tubulin (basal body, red) and acetylated α-tubulin (axoneme, cyan) antibody. The inset shows a high-power image of the basal body and axoneme. DAPI staining is used as counterstaining. Scale bars, 10 μm. (c) ALP activity of IFT80f/f and IFT80d/d OPCs at day 7 of OS induction with or without Shh stimulation (n = 4, triplicates per group). (d) Alizarin Red staining at day 14 in OS culture. IFT80f/f and IFT80d/d OPCs were transfected with BMP2 or Runx2 adenovirus (n = 4, triplicates per group). Primary cilia were stained with Ace-tubulin/γ-tubulin/DAPI. (e) Reporter assay showing 8xGli-responsive luciferase (p8xGli-Luc) activity in IFT80f/f and IFT80d/d OPCs with or without Shh stimulation (n = 3, triplicates per group). (f) Reporter assay showing 8xGli responsive luciferase (p8xGli-Luc) activity in IFT80f/f and IFT80d/d OPCs with or without Shh stimulation (n = 3, triplicates per group). (g) QPCR results showing Ptc1 and Gli1 expression in IFT80f/f and IFT80d/d OPCs with or without Shh stimulation (n = 4, triplicates per group). (h) ALP activity of IFT80f/f and IFT80d/d OPCs transfected with BMP2 or Runx2 adenovirus. Both cells were induced for osteogenesis for 7 days (n = 4, triplicates per group). (i) Alizarin Red staining at day 14 in OS culture. IFT80f/f and IFT80d/d OPCs were transfected with BMP2 or Runx2 adenovirus (n = 4, triplicates per group). (j) QPCR result showing osterix and osteocalcin expression with BMP2 or Runx2 adenovirus transfection in IFT80f/f and IFT80d/d group (n = 4, triplicates per group). Data points indicate the means, while error bars represent s.e.m. Data were analysed using two-way ANOVA followed by Tukey’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.001. NS, not statistically significant.

canonical to non-canonical transduction thereby inhibiting OB differentiation. To test this hypothesis, we first determined whether the effect of IFT80 on stress fibre and RhoA activity is Hh-dependent in OBs. We found RhoA activity increased 1.5-fold in IFT80f/f OPCs with Shh stimulation for 20 min, suggesting that OPCs can respond rapidly to Shh via the Hh non-canonical pathway (Fig. 4d). RhoA activity was greatly promoted by Shh and increased by 1.9-fold in IFT80d/d OPCs. Thus, these results demonstrated that deletion of IFT80 overactivates the non-canonical Hh–RhoA signalling pathway. To get further insight into the mechanism, we then tested whether this non-canonical Hh–RhoA signalling transduction is Smo dependent by using Smo inhibitors—SANT-1 and cyclopamine. Consistent with other studies38,39, SANT-1 and cyclopamine significantly inhibited Hh–Gli signalling transduction in both IFT80f/f and IFT80d/d OPCs (Supplementary Fig. 7a). Both SANT-1 and cyclopamine inhibited RhoA activity in response to Shh stimulation (Fig. 4e), indicating that Hh–RhoA transduction is Smo
Deletion of IFT80 elevates non-canonical Hh–RhoA signalling transduction in OPCs. (a) IFT80^f/f and IFT80^d/d OBs were stained to visualize actin (red) and nucleus (blue). Scale bars, 100 μm. (b) Graph quantifying the percentage of stress fibres shown in a (n = 3 with at least 15 cells analysed). (c) Activated RhoA was detected by ELISA-based G-LISA assay during osteogenesis at indicated time points (n = 4, triplicates per group). (d) G-LISA assay showing RhoA activation in IFT80^f/f and IFT80^d/d OBs treated with Shh for 20 min (n = 4, triplicates per group). (e) G-LISA assay showing RhoA activation in IFT80^f/f and IFT80^d/d OPCs. Both OPCs were treated with Smo inhibitors SANT-1 and cyclopamine (CYC) and then stimulated with Shh for 20 min (n = 4, triplicates per group). (f) G-LISA assay showing RhoA activation in IFT80^f/f and IFT80^d/d OPCs. Both OPCs were treated with PTX (Gzix inhibitor) or C3 (RhoA inhibitor) and then stimulated with Shh for 20 min (n = 4, triplicates per group). (g) Western blot analysis of phospho-MLC2 (p-MLC2) and phospho-Cofilin (p-Cofilin) in IFT80^f/f and IFT80^d/d OPCs. The levels of p-MLC2 and p-Cofilin are normalized to total MLC2 and Cofilin (n = 3). Data points indicate the means, while error bars represent s.e.m. Data were analysed using two-way ANOVA followed by Tukey’s multiple comparison test. *P<0.05, **P<0.01, ***P<0.0001.

Inhibition of non-canonical Hh signalling promotes osteogenesis. To test whether elevated Hh–RhoA activity in IFT80^d/d cells inhibits OB differentiation, RhoA inhibitor—C3 and ROCK inhibitor—Y-27632 were used to suppress RhoA and ROCK activation. Treatment of IFT80^d/d OPCs with C3 or Y-27632 greatly restored ALP activity (Fig. 5a), suggesting that over-activated RhoA signalling is another cause of defective OB differentiation in IFT80^d/d OPCs. To further confirm the role of RhoA in OB differentiation, IFT80^f/f and IFT80^d/d OPCs were stably transfected with dominant negative RhoA (Rhoa19DN) and constitutively active RhoA (Rhoa63CA) vectors and then induced for OB differentiation. Western blot confirmed the similar expression level of Rhoa19DN and Rhoa63CA in both IFT80^f/f and IFT80^d/d OPCs (Fig. 5b). As expected, dominant negative RhoA inhibited stress fibre formation while constitutively active RhoA promotes stress fibre formation (Supplementary Fig. 8a,b). Dominant negative RhoA promoted ALP activity, the formation of Alizarin Red-positive nodules and the expression of osteocalcin (Figs. 5c–e) in both IFT80^f/f and IFT80^d/d OPCs; however, the expression of dominant negative RhoA partially restored OB differentiation in IFT80^d/d OPCs, suggesting that RhoA–stress fibre pathway might be a parallel pathway to cooperate with Hh–Gli pathway to regulate OB differentiation.

CytoD is an F-actin destabilizer by binding to the plus end of F-actin to block the polymerization and the elongation of actins. CytoD treatment significantly restored ALP activity and greatly promoted Alizarin Red accumulation in the extracellular matrix in both IFT80^f/f and IFT80^d/d OPCs (Fig. 5f,g). CytoD treatment also markedly promoted BMP2, Runx2, osteocalcin and osteocalcin expression in both IFT80^f/f and IFT80^d/d OPCs (Fig. 5h), indicating the increase of stress fibres in IFT80^d/d OPCs inhibits OB differentiation.

Inhibition of non-canonical Hh promotes ciliogenesis. Several studies have shown that actin cytoskeleton regulates ciliogenesis as actin cytoskeleton destabilization promotes ciliogenesis. Therefore, we hypothesized that inhibition of non-canonical Hh–RhoA–stress fibre pathway by disrupting stress fibre could restore the ciliogenesis and rescue canonical Hh–Gli signalling transduction in IFT80^d/d OPCs. As expected, the cells treated with C3 and Y27632 showed a significantly reduction of stress fibres in both IFT80^f/f and IFT80^d/d OPCs (Fig. 6a,b). Although the percentage of ciliated cells was not significantly increased in C3 dependent. To further confirm this conclusion, C3 (RhoA inhibitor) and Pertussis toxin (PTX, Gzix inhibitor) were used to inhibit RhoA and its upstream regulator—Gzix in non-canonical Hh signalling pathway. The results showed that PTX and C3 significantly dampened RhoA activation induced by Shh (Fig. 4f). ROCK, a downstream effector of RhoA, inactivates Cofilin, an actin depolymerizing protein, by phosphorylation. ROCK promotes MLC2 phosphorylation through inactivating MLC phosphatase. Both downstream effectors of ROCK regulate the stress fibre formation. Since RhoA activity was overactivated in IFT80^d/d OPCs, we measured the phosphorylation of MLC2 and Cofilin. As we expected, without Shh stimulation, there was no significant difference in phosphorylation of MLC2 and Cofilin between IFT80^f/f and IFT80^d/d OPCs. In contrast, the phosphorylation levels were significantly increased in IFT80^d/d OPCs with Shh stimulation (Fig. 4g). Moreover, inhibition of RhoA activity by C3 significantly inhibited Shh-induced phosphorylation of MLC2 and Cofilin (Fig. 4g). Collectively, these results suggest that deletion of IFT80 overactivates non-canonical Hh signalling pathway showing increasing RhoA activity and stress fibre formation.
and Y27632 treated group, the cilia length is increased along with the actin destabilization (Fig. 6a,c). Even in IFT80df/d OPcs group, those ciliated cells elongated in response to the C3 and Y27632 treatment. To further study the relationship between ciliogenesis and non-canonical Hh signalling, siRNAs were used to inhibit ROCK1 and ROCK2 expression in OPcs (Fig. 6d). siRNAs targeted against ROCK1 or ROCK2 alone slightly promoted cilia elongation; however, siRNA target both ROCK1 and ROCK2 greatly restored the cilia formation in IFT80df/d OPcs (Fig. 6e,f), suggesting both ROCK1 and ROCK2 are involved in actin cytoskeleton related cilia formation. Some evidence showed that the perturbation of actin dynamics by CytoD promotes ciliogenesis and cilia elongation \(45,46\). IFT80df/d and IFT80df/d OPcs were then treated with CytoD for 1 h, and stained for F-actin and cilia. The results showed that stress fibres were disrupted in both IFT80df/d and IFT80df/d OPcs (Fig. 6g,h). CytoD treatment led to a marked elongation of cilia in IFT80df/d group (Fig. 6i,j). Moreover, CytoD treatment partially, but significantly, rescued defective ciliogenesis in IFT80df/d OPcs (Fig. 6i). The average cilia length in DMSO-treated IFT80df/d OPcs was 3.15 μm,
while CytoD treatment increased the cilia length to 6.24 μm (Fig. 6j). In IFT80f/f group, CytoD treatment increased cilia length from 0.8 to 3.6 μm (Fig. 6j). CytoD treatment did not significantly alter the percentage of ciliated cells in IFT80f/f group, but increased the percentage of ciliated cells from 23 to 51% in IFT80f/f group (Fig. 6j). These data suggest that the disruption of stress fibre could rapidly promote cilia formation in IFT80f/f OPCs.

Since CytoD partially disrupted stress fibres and restored cilia formation in IFT80f/f OPCs, we next analysed whether canonical
Deletion IFT80 disrupts the ciliary localization of Smo. Since deletion of IFT80 blocked canonical Hh signalling and over-activated non-canonical Hh signalling, and two pathways share Smo function, we next studied how IFT80 is involved in Smo translocation by immunofluorescence staining for Smo. The result showed that Shh or purmorphamine treatment led to Smo trafficking to cilia (Fig. 7a,b). Loss of IFT80 caused cilia loss and in turn resulted in the widespread cytoplasmic expression of Smo even with Shh or purmorphamine stimulation. Moreover, we found that in IFT80+/OPCs, Hh signalling activation elevated Ptc1 and Gli1 expression and promoted RhoA activation (Fig. 7c,d). However, in IFT80−/−OPCs, Shh or purmorphamine slightly promoted Ptc1 and Gli1 expression but greatly activated RhoA compared with IFT80+/OPCs (Fig. 7c,d), suggesting ectopic activated Smo promotes both canonical and non-canonical Hh pathways, however, it favours non-canonical Hh–RhoA pathway under cilia absent condition. To further seek the reason of this preference, we tested whether the decrease of Smo translocation to cilia enhances Smo binding with Gz1. The results showed that purmorphamine stimulation increased the recruitment of Smo to Gz1 binding in both IFT80+/ and IFT80−/−OPCs (Fig. 7e). However, the binding of Smo and Gz1 were almost doubled in IFT80−/−OPCs compared with IFT80+/ with purmorphamine stimulation for 10 min or 240 min, respectively, suggesting the altered Smo ciliary translocation promotes the Smo and Gz1 binding, which favors non-canonical Hh signalling activation in IFT80−/−OPCs.

Discussion

Although ciliary IFT proteins participate in the formation and homeostasis of various organs, their roles in OB differentiation and bone development are not well-addressed. Our study reveals for the first time that non-canonical Hh signalling negatively regulates OB differentiation and that IFT80 is essential for the balance of the canonical and non-canonical Hh signalling pathways during OB differentiation and bone formation.

Interesting findings have showed that hypomorphic levels of IFT80 in mouse led to embryonic lethality associated with severe bone defects and disrupted Hh signalling47. However, it is unclear if the abnormal bone phenotype results from the direct effect of IFT80 mutation on bone development and/or homeostasis or the indirect effect of IFT80 mutation in other human tissues. Our results showed the mice with deletion of IFT80 in OPCs using OSX-Cre transgenic line shows growth retardation with dramatically decreased bone mass and impaired OB differentiation and proliferation, demonstrating that IFT80 directly affects OB differentiation, proliferation and bone development. In addition, weak OSX expression has been found in the hypertrophic chondrocytes35, thus, IFT80 might also be...
deleted in part of the hypertrophic chondrocyte lineage in OSX;IFT80f/f mice, contributing to the enlarged hypertrophic chondrocyte zone in growth plate. In our previous study, we have found that IFT80 is critical for chondrocyte differentiation by regulating Hh and Wnt signalling48,49. Deletion of IFT80 in chondrocytes with inducible Col2a1-Cre causes reduced length of growth plate in both embryonic stage and postnatal stage50. The difference of cartilage phenotype observed in OSX;IFT80+/+ and Col2a1;IFT80+/+ mice might be due to the different expression pattern and level of OSX and Col2a1 in cartilage. Therefore, the exact role of IFT80 in regulating cartilage formation needs further study.

Shh rapidly promoted RhoA activation within 20 min in both IFT80+/+ and IFT80−/− OPCs via a Gz1-dependent pathway (Fig. 4d,i), while Hh-induced canonical Gli transduction response usually takes hours29, suggesting that Hh–Gz1–RhoA signalling occurs in OPCs, and that the rapid Hh–Gz1–RhoA signalling in OPCs is Gli-independent Hh signalling. Some studies have shown that this ligand-dependent non-canonical Hh signalling regulates actin cytoskeleton in endothelial cells, fibroblasts and cancer cell lines50,50−52. However, it is the first time that we identified this Gli-independent, but Smo-dependent non-canonical Hh signalling also exists in OPCs.

It has been reported that non-canonical Hh pathway participates in the chemotaxis of cholangiocarcinoma cells52, in the cytoskeletal rearrangement of mouse mesenchymal fibroblasts50, in the pro-angiogenic responses of endothelial cells30 and in the migration of fibroblasts29. Here we uncovered an antagonistic role of non-canonical Hh–RhoA signalling pathway in OB differentiation. Deletion of IFT80 elevated RhoA activity and downregulated BMP2-Smad1/5/8 signalling, which greatly inhibited OB differentiation. Inhibition of RhoA or its downstream effector ROCK restored ALP activity of IFT80−/− OPCs. This is supported by the findings that activated RhoA signalling inhibits OB differentiation both in vivo and in vitro53,54. Although RhoA negatively regulates BMP2-induced Smad1/5/8 phosphorylation in a ROCK-independent way55, inhibition of ROCK activity by Y-27632 also promotes OB differentiation54,56,57. Activation of the non-canonical Hh pathway in endothelial cells promotes actin stress fibre formation50. Consistently, we found that overactivation of non-canonical Hh–RhoA signalling increased stress fibre density in OPCs (Fig. 4a,b).

Recently, a functional genomic screen using RNAi uncovered the regulatory role of actin cytoskeleton in ciliogenesis52. Inhibiting actin assembly promotes cilia formation by stabilizing the pericentrosomal preciliary compartment storing, which stores transmembrane proteins destined for cilia52. Inhibiting branched actin network formation by a microRNA mir-129-3p promotes ciliogenesis58. Very recently, actin modification and activation within or outside cilia remains unknown. Whether Smo undergoes differential post-translational modification and activation within or outside cilia remains unknown. How Smo subcellular distribution is maintained and how this is related to cilia and IFT proteins need further studies.

In summary, our data demonstrated that deletion of IFT80 causes cilia shortened or lost. Without an intact cilium structure, activated Smo cannot move into the cilium to activate Gli, thereby inhibiting BMP2 and Runx2 signalling and OB differentiation. Meanwhile, extraciliary Smo coupled Gz1 to overactivate RhoA signalling and then generate excess stress fibres, which also inhibited OB differentiation. Thus, this study reveals a new mechanism by which IFT80 plays a vital role in cilia formation and OB differentiation and is essential for the balance between canonical and non-canonical Hh pathways. In OPCs, the primary cilium provides a specialized space for accumulation of components for the canonical Hh signalling pathway. With Hh stimulation, Smo translocates to the cilium, activates Gli and subsequently drives OB differentiation by regulating BMP2 and Runx2 expression. Meanwhile, activated Smo also couples with Gz1 to activate RhoA, remodelling the cytoskeleton and also providing a negative feedback to canonical Hh signalling (Fig. 7f).

Currently, there are no effective approaches to treat bone disorders caused by mutation of IFT and other ciliary proteins.
Our results reveal a new mechanism regarding the role and mechanism of IFT80 in OB differentiation and bone formation, and highlight IFT80 and non-canonical Hh signalling as therapeutic targets for craniofacial and skeletal abnormalities.

**Methods**

**Generation of IFT80 conditional knockout mice.** An IFT80 gene target vector was constructed with a 5.59-kb left arm homology, a Frt-flankedNeo reporter gene and LacZ gene, a lox-flanked exon 6 of IFT80 and a 4.6-kb right arm homology. Following sequence confirmation of the construct, the targeting vector was linearized and electroporated into the C57BL/6N mouse embryonic stem (ES) cells, followed by positive selection with G418 (for Neo). Three independent ES mutant clones were confirmed by Southern blot and used for generation of chimeric mice by microinjection into blastocysts of C57BL/6j mice. Chimeric mice were bred with wild-type C57BL/6j mice for generation of germ line transmission of the targeted allele. F1 mice were genotyped by PCR using tail genomic DNA samples for the presence of the target IFT80floxed allele. Heterozygous mice were bred with FLP/Frt mice (expressing the Fpe recombinase) to remove the LacZ and Neo cassette and to selected the mice with the IFT80floxed allele. Heterozygous IFT80f/f mice were intercrossed and F2 progeny were genotyped for homozygous IFT80f/f. The homozygous IFT80f/f mice were born at expected Mendelian ratio and did not exhibit any abnormalities.

To specifically inactivate IFT80 in the OB lineage, we crossed the IFT80f/f mice with Oto4-Cre transgenic mice (also known as OSX-GFP-Cre; Ex035), which express Cre under the promoter of osterix. In this transgenic line, Cre mainly targets the OPCs35. The Cre transgene was detected using two primers CreF (5'-CCCTGGAGCTACAGAGAG-3') and CreB (5'-GGCGCGGCAACACCATTTTT-3'). The floxed allele was genotyped from tail genomic DNA for the wild-type allele. Genotyping of mice was performed by PCR using proteinase K-digested tail DNA.

All studies and procedures performed on mice were approved by the University at Buffalo Institutional Animal Care and Use Committee.

**Primary OPCs culture and differentiation.** Primary OPCs were isolated from mouse calvarial bone (4–6 pups of either sex, postnatal day 3) by serial digestion method. Briefly, calvarial bone was dissected and subjected to sequential digestions in collagenase type I (2 mg ml\(^{-1}\), EMD, Darmstadt, Germany), Trypsin (0.25%, Corning, Manassas, VA) and collagenase type I (2 mg ml\(^{-1}\)) for 30 min each. Then the bone chips were subjected to collagenase type I (2 mg ml\(^{-1}\)) again for 10 min. Cells from this digestion were spun down and plated in α-MEM containing 10% foetal bovine serum (FBS), 100 U ml\(^{-1}\) penicillin and 1 μg ml\(^{-1}\) streptomycin. OPCs from IFT80f/f mice were infected with adenovirus, which overexpress either Cre (Ad-CMV-Cre, #1405, Vector Bios) or GFP (Ad-GFP, #1060, Vector Bios). Ad-CMV-Cre infection yielded 95% deletion of IFT80 allele. F1 mice were genotyped by PCR using tail genomic DNA samples for the presence of the target IFT80floxed allele. Heterozygous mice were bred with FLP/Frt mice (expressing the Fpe recombinase) to remove the LacZ and Neo cassette and to selected the mice with the IFT80floxed allele. Heterozygous IFT80f/f mice were intercrossed and F2 progeny were genotyped for homozygous IFT80f/f. The homozygous IFT80f/f mice were born at expected Mendelian ratio and did not exhibit any abnormalities.

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**ROhA activity assay.** Relative RhoA activity was determined using a commercially available ELISA-based RhoA activity assay kit (G-LISA, BK142; Cytoskeleton Inc., Denver, CO, USA) according to the manufacturer’s instructions. Briefly, treated cells were lysed and snap-frozen in liquid nitrogen. Approximately 30–40 μg total protein was used for each sample. The active GTP-bound form of RhoA was detected using a specific antibody. Absorbance readings were obtained at 490nm. Densitometry was performed using the ImageJ software. The experiments were repeated 4–5 times.

**Modulators of Hh signalling and stress fibres.** Purnormorphine (Tocris Bioscience, 4551) is a pharmacological activator of Smo. Purnormorphine was dissolved in DMSO and added to the cell culture at a concentration of 2 μM. Cyclopamine (Tocris Bioscience, 1623) and SANT-1 (Tocris Bioscience, 1974) were dissolved in ethanol and DMSO, respectively. The final concentration used for cells was 5 μM of cyclopamine and 100 μM of SANT-1.

Cell permeable form of C3 transferrase (Cat# CT04, Cytoskeleton), covalently linked to a proprietary cell-penetrating moiety, was used to inhibit RhoA activity. PTX (Tocris Bioscience, 3097) was used to inhibit Gαi. CytoD (Sigma, C2618) and DMSO (used as control, Sigma) were used to induce transient actin cytoskeletal change. For stress fibre staining, cells were treated with 1 μM CytoD for 1 h. For cilia staining, cells were treated with 0.1μM CytoD for 2 h following the serum starving. For osteogenesis, cells were treated with 1 μM CytoD for 1 h and, immediately after the treatment, the cells were washed twice with PBS and then incubated with OS medium. This CytoD treatment was repeated every 4 days.

ROCK silencing was performed in OPCs using validated siRNA oligonucleotides that target endogenous ROCK1 (Santa Cruz, sc-36432) and ROCK2 (Santa Cruz, sc-36433). Scrambled siRNA (Santa Cruz, sc-70077) were included as controls. About 10 μmol siRNA (for 1 well in 24-well plate) were transfected by RNAimax (Invitrogen) according to the manufacturer’s protocol. Efficiency of knockdown was analysed by western blot.

**Stress fibre staining and quantification.** OPCs were washed twice with PBS and fixed in 3.7% formaldehyde in PBS for 10 min at room temperature. Cells were washed and permeabilized with acetone at –20 °C for 3 min. To label stress fibre, cells were blocked with 1% BSA and stained with Rhodamine–phallodin (1:100, Invitrogen) solution for 20 min at room temperature. DAPI (6-diamidino-2-phenylindole, Invitrogen) was used as a counter stain of nuclei. Quantifications were obtained from multichannel images using ImageJ to assess each cell image for its actin stress fibre density66,67. The F-actin images were normalized for average pixel intensity and intensity-thresholded to minimize background fluorescence. ImageJ software was used to generate line profiles. The peaks were counted and the data were expressed as number of stress fibres per cell. The experiment was conducted in triplicate.

**Immunofluorescence.** To visualize cilia structure, immunofluorescence was performed using primary acetylated α-tubulin antibody (1:500, T6793, Sigma) and primary γ-tubulin antibody (1:500, T3320, Sigma). Briefly, cells were serum starved for 48 h and washed with PBS and fixed with 4% paraformaldehyde at room temperature. Fixed cells were permeabilized with 0.03% Triton X-100 and then incubated with the primary antibodies overnight at 4 °C. Alexa Fluor 568-conjugated anti-rabbit (1:1,000, A-11011, Invitrogen) or Alexa Fluor 647-conjugated anti-mouse (1:1,000, A-21235, Invitrogen) antibodies were used as secondary antibody. Counter stain of nuclei was done with DAPI. The experiment was conducted in quadruplicate.

The same procurer was used for Ki67 (Anti-Ki67, 1:300, ab16667, Abcam) staining and Smo staining. To determine Smo localization, cells were co-stained with K67 (Anti-Ki67, 1:300, ab16667, Abcam) and acetylated α-tubulin antibody.
MTS assay. Cells were seeded at 5,000 cells per well in 96-well plate. The MTS assay was performed using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) after 24, 48 and 72 h in culture. The optical density was measured at 490 nm.

Whole-mount skeletal staining. Whole-mount skeletal staining with Alizarin Red and Alcian Blue was performed as previously reported with slight modification. Briefly, mice were fixed in 90% ethanol for at least 1 week. Prior to staining, skin and viscera were removed. Alcian Blue solution (0.01%, in 80% ethanol and 20% glacial acetic acid) was used to stain the cartilage. The skeletons were cleared with 1% KOH solution for 2–3 days and transferred to 1% Alizarin Red S solution (in 1% KOH) for 2–3 days to stain mineralized bone. The skeletons were then cleared with an increased glycerol solution in 1% KOH, and stored in 100% glycerol for image acquisition.

Histology and safranin O staining. Miceibiae were excised, fixed for 24 h in 10% natural buffered formalin, and decalcified in 10% EDTA for 1–2 weeks at 4 °C. The samples were embedded in paraffin, sectioned for 5 μm and stained with H&E. Deparaffinized slides were stained with 0.1% safranin O solution for 5 min to visualize cartilage. Fast green was used as a counter stain.

Histomorphometric analyses. Mice were i.p. injected with calcine (25 mg/kg−1) at Day 30 and Day 34. Mice were killed 2 days after last injection and tibia were dissected, fixed, dehydrated and embedded undecalced in a methacrylate. Longitudinal sections (5 μm) were cut from the centre of the proximal tibiae with a microtome (model 2065; Reichert Jung, Heidelberg, Germany). All measurements were performed with the OsteoMeasure Analysis System (Osteometrics, Atlanta, GA).

Bone micro-CT analysis. A quantitative analysis of the gross bone morphology and microarchitecture was performed with the Micro-CT system (USA) Grand Forks Human Nutrition Research Center, Grand Forks, ND). Femurs from 1-month-old OSX:IFT80f/f mice and OSX:IFT80f/f mice were fixed, scanned and reconstituted as three-dimensional images. Cancellous bones were evaluated in the distal femur metaphysis. About 125 slices (1.5 mm) of bone were measured to determine BV/TV (%), Tb.N (1/μm), Tb.Th (μm) and trabecular spacing (Tb.Sp, μm).

Quantitative PCR. Total RNA was extracted from cultured OBs with Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. cDNA was synthesized from 3 μg total RNA by reverse transcription (cDNA Premix kit (Clontech, Palo Alto, CA). qPCR was performed on the ABI PRISM 7500 real-time PCR machine (invitrogen) with SYBR Green PCR master mix (Invitrogen). Primers were designed with IDT SCI primer design tool (Integrated DNA Technologies, San Diego, CA). Sequence and product length for each primer pair were listed in Supplementary Table 1. All reactions were run in triplicate and determined by the 2−ΔΔCt method.

Western blot. Western blot was performed to detect IFT80 expression using rabbit anti-IFT80 antibody (1:400, PAB15842, Abnova). Calf cartilage was harvested with NP40 lysis buffer (150 mM NaCl, 50 mM Tris pH 7.4 and 1% NP40). Total cell lysates and 2 μg Gzi antibody (sc-136478, Santa Cruz) were incubated for 1 h at 4 °C followed by adding protein A and protein G agarose beads (Santa Cruz) with overnight immunoprecipitation. Immunoprecipitation products were washed and boiled for 10 min for western blot.

Statistical analysis. All data are presented as mean ± s.e.m. (n ≥ 3). Student’s t-test for the comparison between two groups or two-way ANOVA followed by Tukey’s multiple comparison tests was performed. P < 0.05 was considered to be significant. The program GraphPad Prism (Graphpad Software, Inc., San Diego, USA) was used for these analyses.

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Author contributions
S.Y., X.C., R.S. and J.Q designed the research and analysed the data. X.Y., J.C. and X.H. designed, performed experiments and analysed the data. Additional information

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