Canonical and noncanonical intraflagellar transport regulates craniofacial skeletal development

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The primary cilium is a cellular organelle that coordinates signaling pathways critical for cell proliferation, differentiation, survival, and homeostasis. Intraflagellar transport (IFT) plays a pivotal role in assembling primary cilia. Disruption and/or dysfunction of IFT components can cause multiple diseases, including skeletal dysplasia. However, the mechanism by which IFT regulates skeletogenesis remains elusive. Here, we show that a neural crest-specific deletion of intraflagellar transport 20 (IFT20) in mice compromises ciliogenesis and intracellular transport of collagen, which leads to osteosclerosis in the facial region. Whereas platelet-derived growth factor receptor alpha (PDGFRα) was present on the surface of primary cilia in wild-type osteoblasts, disruption of IFT20 down-regulated PDGFRα production, which caused suppression of PDGFR-α signaling, resulting in decreased osteogenic proliferation and increased cell death. Although osteogenic differentiation in cranial neural crest (CNC)-derived cells occurred normally in Ift20−/− mutant cells, the process of mineralization was severely attenuated due to delayed secretion of type I collagen. In control osteoblasts, procollagen was easily transported from the endoplasmic reticulum (ER) to the Golgi apparatus. By contrast, despite having similar levels of collagen type 1 alpha 1 (Col1a1) expression, Ift20 mutants did not secrete procollagen because of dysfunctional ER-to-Golgi trafficking. These data suggest that in the multipotent stem cells of CNSs, IFT20 is indispensable for regulating not only ciliogenesis but also collagen intracellular trafficking. Our study introduces a unique perspective on the canonical and noncanonical functions of IFT20 in craniofacial skeletal development.

Intraflagellar transport (IFT) proteins are required for the assembly of primary cilia (1, 2), which coordinate signaling pathways that are critical for governing cell proliferation, differentiation, survival, and homeostasis (3, 4). Mutations in ciliary proteins result in a diverse set of clinical disorders, termed ciliopathies, which include a wide variety of skeletal phenotypes (5, 6). IFT proteins organize into two complexes, IFT-A and IFT-B (2, 7). IFT-B proteins mediate anterograde transport from the cell body to the cilium tip, whereas IFT-A proteins regulate retrograde trafficking. Genes encoding IFT-A proteins are commonly altered in skeletal ciliopathies. For example, IFT122, IFT40, and IFT44 are frequently mutated in Sensenbrenner and Jeune syndromes, indicating that all six IFT-A components are linked to skeletal ciliopathies (8–10). Conversely, with the exception of IFT80 and IFT172 in Jeune asphyxiating thoracic dystrophy (11, 12), the function of IFT-B complexes in skeletal diseases is unknown.

Intraflagellar transport 20 (IFT20) is the smallest IFT protein in the IFT-B complex and interacts with IFT57/Hippi and the kinesin-II subunit KIF3B (13). Conventional Ift57 and Kif3a/Kif3b null mutations in mice lead to early embryonic lethality due to left–right axis defects, accompanied by a loss of nodal cilia (14–16), suggesting that the IFT-B complex is critical for ciliogenesis. Supporting this idea, the deletion of Ift20 in mouse kidney causes a lack of primary cilia and leads to polycystic kidney disease (17). In addition to the traditional role of IFT in cilium assembly, recent studies have found that an IFT-like particle organized by IFT20 is recruited to the immune synapse for T-cell receptor recycling (18, 19). These studies suggest that IFT proteins participate in intracellular membrane trafficking in immune cells; however, it remains unclear how IFT20 governs developmental processes, including skeletal formation, and how it functions in other cell types, such as multipotent stem cells.

Several rare pleiotropic diseases that affect craniofacial skeletal formation show disruption in primary cilia (20, 21), and orofacial-digital (OFD) syndrome and Bardet–Biedl syndrome (BBS) are related to ciliary dysfunction (22, 23). Consistent with the findings in humans, mutations in Ofl1 in mice and zebrafish cause severe craniofacial abnormalities, including cleft palate and shortened Meckel’s cartilage (22, 24). Both patients and mice with a mutant BBS6 gene display similar broad midfacial malformation and nasal hypoplasia (23). Disrupting ciliogenic components in cranial neural crest cells (CNCCs) frequently results in embryonic and/or postnatal craniofacial abnormalities (25, 26), indicating a tight relationship between primary cilia and craniofacial morphogenesis. Because CNCCs are multipotent stem cells essential for forming craniofacial skeletal components (27, 28), it is important to understand how primary cilia function in CNCCs during craniofacial development.

The purpose of this study is to investigate the molecular function of IFT20 in a multipotent stem cell population during craniofacial development. We found that IFT20 plays a vital role in controlling not only ciliogenesis but also the intracellular cranial neural crest cells | intracellular trafficking | intraflagellar transport | PDGF signaling | primary cilia

Significance

Intraflagellar transport (IFT) plays a critical role in assembling primary cilia that mediate growth factor signaling. The disruption or dysfunction of IFT components can generate multiple diseases, including skeletal dysplasia. However, the mechanism by which IFT regulates skeletogenesis remains elusive. In the present study, we show that neural crest-specific deletion of the gene that encodes intraflagellar transport 20 (IFT20) in mice compromises ciliogenesis and the intracellular transport of collagen, leading to osteosclerosis in the face. Our findings highlight a unique function of IFT beyond its role in ciliogenesis during craniofacial development, suggesting that IFT20 is indispensable for the regulation of not only ciliogenesis but also the intracellular trafficking of collagen in the unique multipotent stem cell population of cranial neural crests.

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trafficking of collagen in CNCCs, demonstrating a function of IFT beyond its role in ciliogenesis during facial skeletal formation.

**Results**

**Disruption of Ift20 in Neural Crest Cells Results in Craniofacial Malformation.** To characterize the function of IFT20 in facial development, we disrupted Ift20 in a neural crest-specific manner using the wingless-related MMTV integration site 1 (Wnt1)-Cre driver line (17, 29) (Fig. S1 A and B; Ift20:Wnt1-Cre or cKO hereafter). Neural crest-specific deletion of Ift20 led to severe craniofacial malformation in mice (Fig. 1). Ift20:Wnt1-Cre embryos were born at Mendelian ratios (Table S1), but had hypertelorism and frontonasal dysplasia with 100% phenotypic penetrance (Fig. 1A). Ift20:Wnt1-Cre mice were born dead with no milk spot, indicating that they died shortly after birth due to difficulties in feeding and breathing. Histological analysis revealed that Ift20:Wnt1-Cre embryos had an abnormal expansion of the facial midline and lacked major craniofacial components, including the palatal shelves and a tongue, at embryonic day (E) 18.5 (Fig. 1A and Fig. S1C). Skeletal staining of Ift20:Wnt1-Cre mice found either a loss or a truncation of craniofacial bones (Fig. 1B). The maxillary and mandibular bones and palatal process of the maxilla in Ift20:Wnt1-Cre mice were hypoplastic, whereas the palatal process of the palatine and pterygoid were absent (Fig. 1B). Ift20:Wnt1-Cre mice had malformed nasal and frontal bones, with an opening in the metopic suture (Fig. 1B), suggesting that cranial neural crest (CNC)-derived facial bones were severely compromised. Mice with neural crest-specific inactivation of Kif3a, a member of the kinesin superfamily required for ciliogenesis, also show bifid nasal cartilage, severe cleft palate, and widely separated frontal bones (30, 31), similar to the features observed in our Ift20:Wnt1-Cre embryos. Because IFT20 appears to bridge the kinesin-II complex (KIF3A/KIF3B) (13), the phenotypic analysis of Ift20:Wnt1-Cre mice further reinforces the idea that there is a link between IFT20 and KIF3A, demonstrating that ciliary dysfunction due to IFT components contributes to a range of craniofacial abnormalities.

Because epithelial-mesenchymal interactions are essential in craniofacial morphogenesis (21, 27), we also deleted Ift20 in an epithelial cell-specific manner using the keratin 14 (K14)-Cre driver line (32). Epithelial-specific deletion of Ift20 (Ift20:K14-Cre) did not induce overt abnormalities in the craniofacial regions (Fig. S2), but Ift20:K14-Cre mice displayed abnormal skin phenotypes, including fewer hair follicles than control mice (Fig. S3). These results suggest that IFT20 is indispensable for facial development in CNC-derived mesenchymal cells, but not in epithelial cells. In this study, we focused on the analysis of the molecular mechanisms of IFT20 in CNC-derived bones during skull development.

**IFT20 Assembles the Primary Cilia That Induce PDGF Signaling Required for Osteogenic Proliferation and Survival During Skull Formation.** Because IFT20 is important for primary cilia assembly...
(17, 33), we attempted to identify the primary cilia, using immunohistochemistry, in control and Ift20:Wnt1-Cre embryos. In control embryos at E12.5, CNC-derived osteogenic cells, which were labeled with RUNX2, a key regulator of osteoblasts, had primary cilia (Fig. 2A, Left). On the other hand, Ift20:Wnt1-Cre embryos had no primary cilia in their CNC-derived osteogenic cells (Fig. 2A, Right), demonstrating that IFT20 is essential for ciliogenesis in CNC derivatives.

Ciliary defects lead to craniofacial abnormalities due to disruptions in Hedgehog signaling (8, 23, 30). However, it remains elusive whether ciliary-mediated Hedgehog is the sole signal axis to govern facial bone development. For instance, neural crest-specific Smo (Smo) mice display skull abnormalities (34); therefore, one might predict that Smo in primary cilia transduces the Hedgehog signaling in skull formation. However, it is not known if Smo is present on cilia in CNC derivatives. This notion raises the question of whether the phenotypes in Smo mutants are attributed to the alterations of ciliary-dependent Hedgehog signaling. Importantly, our analyzed Ift20 mutants showed a skull phenotype distinct from the phenotype in other cilia-deficient mice. Whereas neural crest-specific Kif3a mutants display a metopic craniosynostosis with the alteration of Hedgehog signaling (30, 35), Ift20 mutants did not show any premature suture fusions (Fig. 1B). These notions let us hypothesize that other ciliary-dependent signaling, rather than Hedgehog, would be responsible for skull malformation in Ift20 mutants. To address this hypothesis, we examined platelet-derived growth factor (PDGF) signaling in Ift20:Wnt1-Cre embryos.

PDGF receptor alpha (PDGFRα) is localized on the surface of primary cilia in fibroblasts (36). The disruption of PDGFRα-PI3K

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signaling in CNCCs causes cleft palate and separated frontal bones (37). Thus, we hypothesized that PDGFRα on the surface of primary cilia in CNCCs regulates osteogenic proliferation and cell survival during skull formation. To investigate this idea, we established cultures of primary osteoblasts from nasal and frontal bones, which are CNC-derived (27). Consistent with a previous report (36), PDGFRα localized to the primary cilia in control osteoblasts (Fig. 2B); however, due to the absence of primary cilia, it was not observed in Ift20:Wnt1-Cre osteoblasts but was still present on the cell surface (Fig. 2B). Quantitative RT-PCR and Western blot analysis confirmed that the transcriptional and translational levels of PDGFRα were reduced in Ift20:Wnt1-Cre osteoblasts (Fig. 2C–E). To determine whether the loss of PDGFRα in primary cilia attenuated PDGF-driven intracellular signaling, we measured the activation of PDGFRα and the induction of Akt phosphorylation, one of the effectors of PDGF signaling (38). Control osteoblasts stimulated with PDGF ligand [PDGF-AA] robustly activated PDGFRα and induced Akt phosphorylation, but Ift20:Wnt1-Cre osteoblasts did not (Fig. 2F–H). These results suggest that PDGFRα, localized to primary cilia, regulates Akt phosphorylation through PDGFRα activation, which may govern multiple cascades of cellular events in osteoblasts.

To investigate these cellular events, we first evaluated osteogenic differentiation by using preosteoblast markers RUNX2 and Osterix (OSX). At E12.5, the RUNX2-positive area in frontal bone primordia was comparable between control and Ift20:Wnt1-Cre embryos (Fig. S4A). During middle to late gestation, OSX-positive cells in the frontal bone were also equally distributed between the two groups (Fig. S4B). Next, we examined cell proliferation in embryonic skulls using Ki-67 immunochemistry and staining with OSX. Whereas the levels of OSX were normal, Ift20:Wnt1-Cre embryos had reduced cell proliferation compared with controls at E16.5 (Fig. 2I and J). We further analyzed osteogenic cell survival using a TUNEL assay. Compared with controls, there was a significant increase in the amount of cell death in Ift20:Wnt1-Cre embryos (Fig. 2K and L), suggesting that primary cilia-mediated PDGF signaling...
in osteoblasts plays a critical role in regulating both osteogenic cell proliferation and cell survival. Together, these results indicate that IFT20 is essential for primary cilia formation and controls both osteoblast proliferation and survival through PDGF signaling during skull formation.

**Attenuation of IFT20 Does Not Influence Osteogenic Differentiation, but Leads to Abnormal Bone Mineralization in the Skull.** Because IFT20 may regulate the onset of craniofacial skeletogenesis in middle to late gestation, we examined bone mineralization in control and Ift20:Wnt1-Cre embryos at E16.5. H&E staining showed continuous bone tissues in control skulls but not in Ift20:Wnt1-Cre skulls (Fig. 3A). The von Kossa staining revealed discontinuous calcium deposition in Ift20:Wnt1-Cre calvaria (Fig. 3A). OSX and collagen type 1 alpha 1 (Col1a1), both markers of osteoblast differentiation, were also analyzed using immunohistochemistry and section in situ hybridization. Both OSX and Col1a1 were normally produced and expressed in osteogenic tissues in control and Ift20:Wnt1-Cre skulls (Fig. 3A). Quantitative RT-PCR further confirmed that the expression of osteogenic differentiation genes, such as Runx2, Osx, Ibsp, and Ocm, was unchanged in the nasal and frontal bones of Ift20:Wnt1-Cre embryos (Fig. 3B), suggesting that IFT20 is not required for osteogenic differentiation but is necessary for the process of mineralization during skull development.

To address this hypothesis, we examined the process of mineralization using primary osteoblasts. After adding ascorbic acid and β-glycerol phosphate, an inducer of mineralization, both control and Ift20-mutant osteoblasts were examined. The control osteoblasts formed white clusters at 1 wk, becoming solid nodules that positively stained with Alizarin red within 2 wk (Fig. 3C). With Ift20:Wnt1-Cre osteoblasts, no cell clusters developed and few osteogenic nodules stained with Alizarin red (Fig. 3C). Because collagen fibrils are necessary for mineralization when skull bones are formed (39), we visualized the collagen in the cultured osteoblasts. Picrosirius red staining revealed that the amount of collagen produced by Ift20:Wnt1-Cre osteoblasts was less than the amount of collagen produced by control cells (Fig. 3D), and discontinuous collagen fibrils were observed in the skull of Ift20:Wnt1-Cre mice (Fig. 3E). Taken together, these results demonstrate that IFT20 is essential for normal mineralization in the skull.

**IFT20 Controls Procollagen Trafficking from the Endoplasmic Reticulum to the Golgi Apparatus in CNC-Derived Osteoblasts.** Although the transcriptional levels of Col1a1 were comparable between control and Ift20:Wnt1-Cre embryos (Fig. 3A), the amount of collagen in the extracellular matrix was decreased in Ift20:Wnt1-Cre osteoblasts (Fig. 3D and E). These results suggest that Col1a1 was transcribed normally and that procollagen was produced but was not secreted appropriately into the external cellular environment, thus leading to decreased collagen fibril formation. This finding prompted us to hypothesize that IFT20 participates in the intracellular trafficking of procollagen.

We first analyzed the localization of IFT20 in wild-type osteoblasts by co-staining with the cis-Golgi marker GM130. Consistent with a previous report (40), IFT20 localized to the Golgi apparatus (Fig. S5). Staining of calnexin, the marker of endoplasmic reticulum (ER), further confirmed that IFT20 was not present in the ER (Fig. S6). Next, we examined whether the secretion of procollagen in Ift20-mutant osteoblasts was affected by treatment with ascorbic acid. Before ascorbic acid stimulation, the procollagen was smoothly transported into the Golgi in 80% of control osteoblasts. On the other hand, only 40% of Ift20-mutant osteoblasts showed collagen I transition to the Golgi, suggesting that procollagen secretion is slower than for the control cells. Procollagen secretion was still hampered at 3 h in Ift20-mutant osteoblasts. Although there were tendencies to delay procollagen secretion, Ift20-mutant osteoblasts could secrete procollagen to a similar extent as controls after 4 h of ascorbic acid stimulation (Fig. 4 A and B). These data suggest that procollagen transfer to the Golgi was slow at an early phase of collagen secretion, but once the osteoblasts started to secrete collagen I, there were no many differences in intracellular collagen I localization between control and Ift20-mutant osteoblasts. Western blot analysis further verified that the procollagen in Ift20-mutant osteoblasts remained intracellularly much longer than in controls (Fig. 4 C and D), indicating that procollagen secretion is hampered by the dysfunction of ER-to-Golgi trafficking.

To monitor the dynamics of intracellular protein transport from the ER to the Golgi, we used a plasmid encoding vesicular stomatitis virus G protein tagged with EGFP (VSVG-GFP) (41). VSVG-GFP is a powerful tool used in the study of membrane transport because of its reversible misfolding and retention in the ER at 40 °C, and its ability to move out of the ER and into the Golgi upon reducing the temperature to 32 °C (41). We transfected control and Ift20-mutant osteoblasts with the VSVG-GFP plasmid and examined the movement of GFP using immunocytochemical staining with GM130. After temperature reduction from 40 °C to 32 °C, GFP colocalized with GM130 within 5 min in the majority (84%) of control cells (Fig. 4 E and F). On the other hand, in the same time course, only 51% of Ift20-mutant osteoblasts showed colocalization (Fig. 4 E and F).

To gain further insight into the potential cellular mechanisms underlying the defective mineralization in Ift20:Wnt1-Cre embryos, we monitored the ER-to-Golgi transport of VSVG-GFP with time-lapse imaging. Live imaging revealed that GFP accumulated more slowly in the Golgi apparatus of Ift20 mutants than in the Golgi apparatus of control osteoblasts (Movies S1 and S2). Finally, we examined whether the transport of procollagen into the Golgi was affected in Ift20-mutant skulls in vivo. We noted that only osteoblasts near the osteogenic front of wild-type skulls were enriched for procollagen in the Golgi. Therefore, we focused on the area close to the osteogenic front. At E16.5, 55% of wild-type osteoblasts contained procollagen in the Golgi, whereas only 5% of Ift20-mutant osteoblasts had procollagen (Fig. 4 G and H). At E18.5, 50% of Ift20-mutant osteoblasts had procollagen in the Golgi, compared with 70% of wild-type osteoblasts (Fig. 4 G and H), indicating that there is catching up, to some extent, of the delayed ossification in Ift20 mutants at E18.5, which is consistent with in vitro observations. These results suggest that IFT20 is required for the ER-to-Golgi transport of procollagen, which is a critical first step of procollagen intracellular trafficking during skull mineralization.

Taken together, we conclude that IFT20 plays a pivotal role in skull development through (i) the formation of primary cilia, which are required for mediating the PDGF signaling that controls both osteogenic proliferation and cell survival, and (ii) the regulation of the ER-to-Golgi transport critical for collagen secretion during skull development (Fig. 5).

**Discussion**

IFT plays a crucial role in the assembly of primary cilia, which are highly conserved organelles that project from the surface of many cells. However, the details of how IFT governs craniofacial skeletal development remain elusive. In this study, we showed that IFT20 is required for assembling the primary cilia that control skeletogenic proliferation and cell survival through the precise regulation of PDGF signaling during craniofacial formation. We also found a potential link between IFT20 and procollagen trafficking, which may be important for the secretion of collagen. Our study introduces a unique perspective on the canonical and noncanonical functions of IFT20 in craniofacial skeletal formation.
IFT20 Is Essential for Assembling Primary Cilia That Transduce PDGF Signaling During Craniofacial Skull Formation. Growth factor signaling in CNCCs regulates the patterning and growth of facial primordia (21, 27, 28). Although it has been widely recognized that primary cilia are projected like antennas and serve as either chemosensory or mechanosensory organelles, their role in CNCCs remains unclear. Because neural crest-specific loss of Pkd2, which encodes a mechanoreceptor in cilia, leads to multiple craniofacial anomalies at postnatal ages, but not in mice embryos (42), one might speculate that primary cilia predominantly function as chemosensors that mediate numerous signaling pathways during embryonic development (36, 43, 44). Consistent with a previous report analyzing the disruption of IFT20 in kidney development (17), IFT20 was required for the formation of primary cilia in CNC-derived osteogenic cells (Fig. 2 A and B), demonstrating that IFT20 plays an important role in assembling primary cilia that may transduce growth factor signaling in facial development.

During skull development, primary cilia might predominantly participate in the regulation of PDGF signaling, rather than in Hedgehog signaling. Supporting this idea, we found that one of the PDGF receptors, PDGFRα, clearly localized to the cilia surface in primary osteoblasts (Fig. 2B). In addition, we found that PDGF-Akt signaling through primary cilia is required for osteoblast proliferation and survival (Fig. 2B). In agreement with the phenotype of mouse embryos with a CNCC-specific inactivation of Pdgfra (45), the control and Ift20−/−mutant embryos had a similar number of proliferating CNCC-derived osteogenic cells during early-middle gestation, but a much lower number during middle-late gestation in Ift20−/−mutants (Fig. 2F), indicating that PDGF signaling via primary cilia in CNCCs may not be essential at the early stage of osteoblastogenesis, but is required...
for cell proliferation and survival during late-stage skull development. However, we cannot exclude the possibility that Hedgehog signaling may also be involved in the molecular pathogenesis, because dysregulation of Hedgehog signaling has been frequently observed in ciliary mutants displaying skeletal dysmorphogenesis (46, 47). Although the localization of Smo to primary cilia has been identified in nodal cilia (44), a key question would be whether Hedgehog signaling mediators, including Smo, are present in the primary cilia of CNC-derived osteoblasts. This alternative possibility will need to be addressed to understand the synergistic regulatory mechanisms of growth factor signaling (e.g., PDGF and Hedgehog signaling) in primary cilia during skull formation.

**IFT20 Is Critical for Regulating the Trafficking of Procollagen in CNC-Derived Osteoblasts.** Compared with other IFT-B proteins, IFT20 has an unusual distribution. In addition to being found in both primary cilia and the centrosome pool, it is present in the Golgi apparatus (40). This finding raises the possibility that IFT20 might participate in intracellular membrane trafficking, in addition to its role in ciliary assembly. Supporting this hypothesis, recent studies have demonstrated that IFT20 is associated with the trafficking of ciliary membrane proteins from the Golgi to the cilium, opsin trafficking, and membrane trafficking in lymphoid and myoid cells (18, 19, 33, 48); however, it remains unclear whether IFT20 functions as a noncanonical IFT system during craniofacial skeletal development.

*IFT20-Wnt1-Cre* embryos showed immature bone mineralization (Fig. 1), but this phenotype was not simply explained by the down-regulation of osteogenic differentiation activities (Fig. 3). Osteoblastic differentiation genes, including *Col1a1*, were expressed normally; however, *Ifit20*-mutant osteoblasts were not capable of mineralization to the same degree as controls. Our study suggests that this defect is because *Ifit20*-mutant cells cannot secrete procollagen molecules into the extracellular environment within the same time course as controls (Fig. 4), demonstrating that IFT20 may be involved in procollagen trafficking of osteoblasts. Meanwhile, noncollagen factors secreted by bones may also be affected in *Ifit20-Wnt1-Cre* mutants. For example, bioactivity of both bone morphogenetic protein-15 (BMP-15) and growth differentiation factor-9 (GDF-9) is tightly regulated by the Golgi-mediated secretory pathway (49, 50). Therefore, IFT20 might participate in the regulation of the secreting pathway of those growth factors in skull formation; this possibility needs to be clarified in the future.

In humans, mutations in *SEC23A*, a gene encoding a component of the coat protein complex II (COPII), cause cranioleonticulosutural dysplasia (CLSD) (51, 52). CLSD is an autosomal recessive disorder characterized by skeletal defects and separated frontal bones, along with hypertelorism (53), resembling the features of *Ifit20-Wnt1-Cre* embryos. COPII-coated vesicle formation is critical in anterograde protein trafficking from the ER to the Golgi apparatus (54). Whereas procollagen bundles exiting from the ER have a length of 300 nm, a much larger size than COPII can generally transport (39), studies suggest that large procollagen cargo might be transported to the Golgi by a specialized ER-Golgi intermediate compartment, known as the vesicular tubular cluster (VTC) complex, in a COPII-dependent manner (55). Because ER export complexes are composed of arrays of budding vesicles and cytoplasmic VTCs (56), this finding prompted us to hypothesize that IFT20 participates in the composition of VTCs and plays a role in procollagen trafficking from the ER to the Golgi, which is essential for the initial process of procollagen transport. Correspondingly, we found that procollagen transport from the ER to the Golgi was delayed in *Ifit20*-mutant osteoblasts (Fig. 4). VSVG-GFP time-lapse imaging further revealed that the transport machinery was affected in *Ifit20* mutants (Movies S1 and S2), suggesting that IFT20 may be involved in ER export complexes.

The golgin GMAP210/TRIP11 anchors IFT20 to the Golgi complex, and both molecules function together in the trafficking of ciliary membrane proteins (40). An initial report suggests that loss of GMAP210/TRIP11 in mice alters glycosylation in the Golgi, leading to the intracellular accumulation of Perlecan in chondrocytes and resulting in a neonatal lethal form of skeletal dysplasia (57). However, recent studies suggest that GMAP210/TRIP11 is required for efficient membrane trafficking because it regulates vesicle tethering from the ER (56, 58, 59). Therefore, the IFT20–GMAP210 complex in CNC-derived osteoblasts may act in ER-to-Golgi trafficking.

In summary, our study introduces a unique perspective for canonical and noncanonical IFT function in craniofacial skeletal formation. Our findings may also contribute to our understanding of the molecular pathogenesis of skeletal ciliopathies.

**Experimental Procedures**

**Animals.** *Ifit20-flox* mice (17), *Wnt1-Cre* mice (29), and *K14-Cre* mice (32) were obtained from The Jackson Laboratory. All mice were maintained in the animal facility of The University of Texas Medical School at Houston. The experimental protocol was reviewed and approved by the Animal Welfare Committee and the Institutional Animal Care and Use Committee of The University of Texas Medical School at Houston.

**Skeletal Preparations, Histological Analysis, Immunofluorescent Staining, and TUNEL Assays.** Staining of bone and cartilage of embryos with Alizarin red/Alcian blue was carried out as described previously (60). H&E staining, immunofluorescent staining, and TUNEL assays of paraffin sections were performed as described previously (61). The von Kossa staining was performed using a von Kossa staining kit (Abcam; ab150687). Picrosirius red staining
was performed using 1% picrosirius red solution (Sigma-Aldrich; 365548 and P6744). Section in situ hybridization was performed as previously described (62). Primary antibodies used in immunofluorescent staining were as follows: RUNX2 (1:400; Cell Signaling; no. 12556), acetylated tubulin (1:1,000; Sigma-Aldrich; T6793), gamma-tubulin (1:1,000; Sigma-Aldrich; T5326), OSX (1:200; Abbco; ab22552), Ki-67 (1:100, BD Biosciences; 556069), GM130 (1:200, BD Biosciences; 610882), calnexin (1:100, EMD Millipore; MAB3126), and collagen type I (1:100; Cedarlane; CLS0151AP). Slides were viewed with an Olympus Fluoview FV1000 laser scanning confocal microscope using the software FV10-ASW Viewer (version 3.1).

Isolation and Culture of Primary Osteoblasts. We collected calvarial osteoblasts from control and Ift20:Wnt1-Cre embryos at E18.5. Nasal and frontal bones were subjected to five sequential digestions with an enzyme mixture containing 1 mg/mL collagenase type I (Sigma-Aldrich; C0130) and 1 mg/mL collagenase type II (Sigma-Aldrich; C8585). Cell fractions (from two to five of the sequential digestions) were collected and cultured in growth medium (α-MEM supplemented with 10% [vol/vol] FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin) and in osteogenic medium (growth medium supplemented with 50 μg/mL ascorbic acid and 2 mM β-glycerophosphate). For the analysis of PGDFRα signaling, cells were treated with 50 ng/mL PGDF-acetic acid (PeproTech; 315-17).

Western Blot Analysis. We prepared the osteoblast lysates from control and Ift20:Wnt1-Cre embryos at E17.5. The total RNA was extracted with Dnase I (Roche) before cDNA synthesis. From the cultured osteoblasts, total RNA was purified using the RNeasy Plus Mini Kit (Qiagen). cDNA was synthesized using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). Quantitative RT-PCR was carried out using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). The sequences of the specific primer sets are listed in Table S2.

Statistical Analysis. The Student’s t test was used for statistical analysis. A P value of less than 0.05 was considered statistically significant.

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