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IFT20 is required for the maintenance of cartilaginous matrix in condylar cartilage

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Abstract

Condylar cartilage is a joint cartilage essential for smooth jaw movement. The importance of ciliary proteins in condylar cartilage development has been reported. However, little is known about how ciliary proteins control the homeostasis of condylar cartilage. Here we show that intraflagellar transport 20 (IFT20), a ciliary protein, is required for the maintenance of cartilaginous matrix in condylar cartilage. Utilizing *NG2-CreER* mice expressed in condylar cartilage, we deleted *Ift20* by tamoxifen treatment at juvenile-to-adult stages. In wild-type condylar cartilage, IFT20 was robustly produced in the cis-Golgi, but deletion of *Ift20* by tamoxifen induction of *NG2-CreER* (*Ift20:NG2-CreER*) resulted in reduced cell proliferation and decreased Golgi size in condylar cartilage. Importantly, while the primary cilia were present in cartilage cells in the condylar layers of wild-type mice, no primary cilia were present in the *Ift20:NG2-CreER* condylar layers. Consistent with this finding, ciliary-mediated Hedgehog signaling was severely attenuated in *Ift20* mutant chondrocytes, and thus the production levels of type X collagen were significantly reduced in *Ift20:NG2-CreER* mice. These results suggest that IFT20 is required for Golgi size and Hedgehog signaling to maintain cartilaginous matrix in condylar cartilage. Our study highlights the unique function of IFT20 in the homeostasis of condylar cartilage.

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Author contributions

Y.K. designed the study. M.Ki., H.Y., M.E., and M.Ka. performed the experiments. M.Ki., H.Y., M.Ka. and Y.K. analyzed the data. D.C. provided *Col2a1-CreERT2* mice. M.Ki., and Y.K. wrote the manuscript. All authors contributed to the manuscript. Y.K. supervised the project.

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Keywords

Condyle; Chondrocyte; Golgi; Intraflagellar transport; Mice

Introduction

Condylar cartilage is required for smooth jaw movement when chewing and speaking. A series of studies have highlighted the importance of the developmental aspects of condylar cartilage ⁽¹⁾. Particularly, growth factor signals, including Hedgehog, TGF- β /BMP and Wnt, are essential for the regulation of chondrocyte proliferation and differentiation in condylar cartilage ⁽²⁾ ⁽³⁾ ⁽⁴⁾. However, little is known about how exactly these growth factor signals are transduced to maintain the homeostasis of condylar cartilage.

Primary cilia are antenna-like organelles that coordinate multiple signaling pathways, and mutations in genes encoding ciliary proteins lead to a diverse set of clinical conditions called “ciliopathies” ⁽⁵⁾. Importantly, numerous studies have indicated the molecular link between ciliopathies and craniofacial defects ⁽⁶⁾, and these findings suggest that mutations in ciliary protein genes may negatively impact the morphology and/or function of craniofacial joints. In fact, deletion of *Kif3a* in chondrocytes results in abnormal condylar cartilage formation and growth ⁽⁷⁾. However, it remains unclear how ciliary proteins control the maintenance of cartilaginous matrix in condylar cartilage.

Intraflagellar transport (IFT) is the bidirectional transport of multi-subunit protein complexes required for ciliary assembly, and IFT20 is the smallest IFT protein in the IFT-B complex ⁽⁸⁾. In addition to the traditional role of IFT20 in cilium assembly, several studies have shown that IFT20 has a unique function, e.g. in the control of intracellular membrane trafficking, Cbl-mediated ubiquitination, and nucleation of Golgi-derived microtubules ⁽⁹⁻¹¹⁾. In support of these findings, we recently found that both the canonical and non-canonical role of IFT20 in neural crest cells are essential for skull formation ⁽¹²⁾. However, it remains elusive how IFT20 functions in other craniofacial tissues, including condylar cartilage.

The aim of this study is to investigate the role of IFT20 in condylar cartilage, to identify regulatory mechanisms for the maintenance of cartilaginous matrix.

Materials and methods

Animals

The Animal Welfare Committee and the Institutional Animal Care and Use Committee of The University of Texas Medical School at Houston approved the experimental protocol. *Col2a1-CreER^{T2}* mice were generated and maintained as previously described ⁽¹³⁾. *Ift20*-floxed mice ⁽¹⁴⁾, *NG2-CreER* mice ⁽¹⁵⁾, *Aggrecan-CreER^{T2}* mice ⁽¹⁶⁾, and *Rosa26* reporter mice ⁽¹⁷⁾ were obtained from the Jackson Laboratory. Tamoxifen (Sigma; T5648) was administrated intraperitoneally (75 mg/kg) twice a week starting from 5 weeks. We compared *Ift20* conditional knockout (*Ift20^{flox/flox}; NG2-CreER⁺*) mice with wild-type controls (*Ift20^{flox/flox}; NG2-CreER⁻*). These mice were maintained on a mix genetic background.

Histological analysis and immunostaining

Safranin O and X-gal staining was carried out using standard methods. Anti-IFT20 (Proteintech; 13615-1-AP, 1:100), anti-acetylated tubulin (Sigma; T6793, 1:500), anti-gamma tubulin (Sigma; T5326, 1:500), anti-GM130 (BD; 610822, 1:200), anti-Collagen X (Abcam; ab58632, 1:100), and Smoothed (Abcam; ab38686, 1:400) antibodies were used for immunostaining. Cell proliferation was analyzed using a proliferating cell nuclear antigen staining kit (Life Technologies; 931143). Images were captured with an Olympus FluoView 1000 confocal microscope. More than 100 cells in three independent experiments were randomly analyzed and quantified.

Cell culture

ATDC5 cells were cultured using alpha-MEM medium (Sigma-Aldrich; M8042) containing 10% fetal bovine serum (FBS) (Sigma-Aldrich; F4135), 2% L-glutamine (Sigma-Aldrich; G7513), and 1% Penicillin-Streptomycin (Sigma-Aldrich; P4333). After reaching 70% confluency, scramble or IFT20 siRNA (Thermo Fisher Scientific; 120nM) was transfected into ATDC5 cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific; 13778075). To induce ciliogenesis, cells were starved with alpha-MEM medium containing 0.1% FBS for 24 hours. To stimulate Hedgehog signaling, ATDC5 cells were treated with Smoothed agonist (Calbiochem; 566661, 100nM) or Purmorphamine (Calbiochem; 540223, 1,000nM) for 24 hours.

Quantitative real-time RT-PCR

Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific; 15596-026). Quantitative RT-PCR was carried out using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad; 1725274). The conditions for qRT-PCR were 95°C for 2 min, 95°C for 5 sec, and 60°C for 30 sec, for 40 cycles. Primer sequences for *Gli1* were 5'-AGCCCGCTTCTTTGTTAATTTGA-3' and 5'-CCAAGCCAACCTTTATGTCAGGG-3'. Primer sequences for *Ptch1* were 5'-CTTCTCCTATCTTCTGACGGGT-3' and 5'-AAAGAACTGCGGCAAGTTTTTG-3'. Primer sequences for *Gapdh* were 5'-CGTCCCGTAGACAAAATGGT-3' and 5'-TCAATGAAGGGGTCGTTGAT-3'. Data were normalized to *Gapdh* levels and quantified using the 2^{-CT} method.

Western blotting analysis

Cell lysates were separated by SDS-PAGE (Bio-Rad; 4561036). Anti-IFT20 (Proteintech; 13615-AP, 1:1,000), anti-GAPDH (Cell Signaling technology; 14C10, 1:10,000), and Goat anti-rabbit IgG HRP-conjugate (Millipore sigma; 12-348, 1:5,000) antibodies were used for western blotting. The Clarity Max ECL Substrate (Bio-Rad; 1705061) was used for chemiluminescent detection, and the signals were quantified with the image-J software.

Statistical analysis

A two-tailed Student's *t* test was used for the two groups. A *p* value of less than 0.05 was considered statistically significant.

Results and Discussion

Disruption of *Ift20* results in abnormal Golgi size in condylar cartilage

Temporomandibular joint disorder (TMD) is widely recognized as an idiopathic juvenile form of arthritis ⁽¹⁸⁾. To identify a Cre line to address the etiology of TMD from juvenile stages in condylar cartilage in mice, we compared the Cre activity among established chondrocyte-specific Cre mouse lines *Col2a1-CreER^{T2}*, *Aggrecan-CreER^{T2}*, and Neuron glia antigen-2 (*NG2-CreER*). *NG2* (*Cspg4*) encodes a chondroitin sulfate proteoglycan ⁽¹⁹⁾. *NG2-CreER* mice have been used to label glia cells, but this mouse line has not been used for studying condylar cartilage. In our Cre induction schedule starting from 5 weeks, both *Col2a1-CreER^{T2}* and *Aggrecan-CreER^{T2}* directed mosaic Cre-mediated recombination in condylar cartilage (Fig. 1). On the other hand, *NG2-CreER* effectively labeled chondrocytes in all polymorphic, flattened, and hypertrophic layers (Fig. 1). These data suggest that *NG2-CreER* serves as an additional and homogeneous Cre-inducible mouse line to study condylar cartilage. To characterize the function of ciliary proteins in condylar cartilage, we utilized *NG2-CreER* to delete *Ift20* in mice (cKO hereafter). To address the role of IFT20 from juvenile to adult stages, tamoxifen was initiated from 5 weeks of age. Histological analysis showed that the area of the Safranin O-stained layer in cKO mice was smaller to control mice (Fig. 2A). To examine whether the reduction in size of the Safranin O-positive layer was due to decreased numbers of condylar chondrocytes, cell proliferation activity was examined. In cKO mice, a significant reduction in the number of PCNA-positive cells was observed in polymorphic, flattened, and hypertrophic layers (Fig. 2B). Along with the reduction in cell numbers in condylar cartilage (Fig. 2C), the size of condylar chondrocytes in cKO mice was smaller (cell size peak: 50-60µm, red arrow) than in controls (cell size peak: 80-90µm, blue arrow) mice (Fig. 2D). It is known that the Golgi apparatus expands in chondrocytes during the secretion of cartilaginous matrix ⁽²⁰⁾. Therefore, we hypothesized that IFT20 controls the maintenance of Golgi size in condylar cartilage. Interestingly, in control condylar cartilage, IFT20 was robustly expressed in the cis-Golgi (Supplemental Fig. 1). In agreement with the pattern of *NG2-CreER* recombination (Fig. 1), IFT20 was disrupted in all layers of condylar cartilage in cKO mice (Fig. 2E, **left panel**). Interestingly, the Golgi labeled by GM130, a marker of cis-Golgi, was smaller in cKO compared with control mice (control: $9.42 \pm 0.62 \mu\text{m}^2$; cKO: $5.26 \pm 0.77 \mu\text{m}^2$; $P < 0.05$) (Fig. 2E, **right panel**). Because IFT20 can control the nucleation of Golgi-derived microtubules in tumor cells ⁽¹¹⁾, we examined whether IFT20 is capable of regulating Golgi size in chondrocytes. Consistent with our findings *in vivo* (Fig. 2E), the knockdown of IFT20 by siRNA in ATDC5 cells led to a smaller Golgi shape compared with that seen in control cells (Supplemental Fig. 2). These results indicate that IFT20 is required for maintaining Golgi size when chondrocytes secrete cartilaginous matrix in condylar cartilage.

IFT20 is required for ciliary-dependent Hedgehog signaling in condylar cartilage

During craniofacial formation, primary cilia transduce numerous developmental signals such as Hedgehog signaling ⁽⁶⁾. Importantly, mice lacking *Indian Hedgehog* (*Ihh*) in chondrocytes display abnormal condylar cartilages ⁽²⁾ ⁽²¹⁾. This led us to hypothesize that ciliary-dependent Hedgehog signaling may be responsible for the reduction of cartilaginous matrix in cKO mice. To determine whether the loss of primary cilia leads to the attenuation of

Hedgehog signaling, the ATDC5 chondrogenic cell line was used to examine the role of IFT20. Compared with control cells, the numbers of primary cilia-positive cells were reduced in *si-Ift20* cells (Supplemental Fig. 3). To stimulate hedgehog signaling, both control and *si-Ift20* cells were treated with Hedgehog agonists (either SAG or Puromorphine), with control cells showing robust induction of Smoothened (Smo) on the surface of primary cilia (Fig. 3A, **upper panel**). Whereas around 30-40% of *si-Ift20* cells still formed primary cilia (Supplemental Fig. 3), no Smo accumulation was observed in *si-Ift20* cells (Fig. 3A, **middle and lower panel**). To examine whether IFT20-assembled primary cilia transduce Hedgehog signaling, cells were stimulated with Hedgehog agonists, and the expression levels of *Gli1* and *Ptch1* were quantified. Consistent with absence of Smo induction in primary cilia (Fig. 3A), the expression of both *Gli1* and *Ptch1* was significantly downregulated in *si-Ift20* cells (Fig. 3B). Importantly, while primary cilia were abundantly present in condylar cartilage in control mice, no primary cilia were present in the polymorphic, flattened, and hypertrophic layers of *Ift20* cKO mice (Fig. 3C). These results suggest that IFT20 regulates ciliary-dependent Hedgehog signaling in condylar cartilage. Importantly, Hedgehog signaling regulates collagen type X (Col X), a critical cartilaginous matrix component essential for the maturation of chondrocytes ⁽²²⁾. Supporting this concept, the production of Col X was significantly reduced in condylar cartilage in cKO mice (Fig. 3D). Taken together, these results suggest that IFT20-assembled primary cilia transduce Hedgehog signaling, which is required for the production of Col X to maintain the maturation of condylar cartilage.

In summary, utilizing the *NG2-CreER* mouse line, we showed that IFT20 is required for (1) maintenance of the Golgi size, and (2) Hedgehog signaling to preserve cartilaginous matrix in condylar cartilage (Fig. 3E). Our study introduces a unique perspective on the importance of ciliary proteins for condylar cartilage homeostasis, and may contribute to the understanding of the etiology of temporomandibular joint disorders in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Hinton RJ, Jing J, Feng JQ. Genetic Influences on Temporomandibular Joint Development and Growth. *Current topics in developmental biology*. 2015;115:85–109. [PubMed: 26589922]
2. Shibukawa Y, Young B, Wu C, et al. Temporomandibular joint formation and condyle growth require Indian hedgehog signaling. *Developmental dynamics : an official publication of the American Association of Anatomists*. 2007;236(2):426–34. [PubMed: 17191253]
3. Jing J, Hinton RJ, Mishina Y, Liu Y, Zhou X, Feng JQ. Critical role of *Bmpr1a* in mandibular condyle growth. *Connective tissue research*. 2014;55 Suppl 1:73–8. [PubMed: 25158185]

4. Zhou Y, Wang T, Hamilton JL, Chen D. Wnt/beta-catenin Signaling in Osteoarthritis and in Other Forms of Arthritis. *Current rheumatology reports*. 2017;19(9):53. [PubMed: 28752488]
5. Berbari NF, O'Connor AK, Haycraft CJ, Yoder BK. The primary cilium as a complex signaling center. *Current biology : CB*. 2009;19(13):R526–35. [PubMed: 19602418]
6. Brugmann SA, Cordero DR, Helms JA. Craniofacial ciliopathies: A new classification for craniofacial disorders. *American journal of medical genetics Part A*. 2010;152a(12):2995–3006. [PubMed: 21108387]
7. Kinumatsu T, Shibukawa Y, Yasuda T, et al. TMJ development and growth require primary cilia function. *Journal of dental research*. 2011;90(8):988–94. [PubMed: 21566205]
8. Yuan X, Yang S. Primary Cilia and Intraflagellar Transport Proteins in Bone and Cartilage. *Journal of dental research*. 2016;95(12):1341–9. [PubMed: 27250654]
9. Schmid FM, Schou KB, Vilhelm MJ. IFT20 modulates ciliary PDGFRalpha signaling by regulating the stability of Cbl E3 ubiquitin ligases. 2018;217(1):151–61.
10. Finetti F, Paccani SR, Riparbelli MG, et al. Intraflagellar transport is required for polarized recycling of the TCR/CD3 complex to the immune synapse. *Nature cell biology*. 2009;11(11):1332–9. [PubMed: 19855387]
11. Nishita M, Park SY, Nishio T, et al. Ror2 signaling regulates Golgi structure and transport through IFT20 for tumor invasiveness. 2017;7(1):1.
12. Noda K, Kitami M, Kitami K, Kaku M, Komatsu Y. Canonical and noncanonical intraflagellar transport regulates craniofacial skeletal development. *Proceedings of the National Academy of Sciences of the United States of America*. 2016;113(19):E2589–97. [PubMed: 27118846]
13. Chen M, Lichtler AC, Sheu TJ, et al. Generation of a transgenic mouse model with chondrocyte-specific and tamoxifen-inducible expression of Cre recombinase. *Genesis (New York, NY : 2000)*. 2007;45(1):44–50.
14. Jonassen JA, San Agustin J, Folliot JA, Pazour GJ. Deletion of IFT20 in the mouse kidney causes misorientation of the mitotic spindle and cystic kidney disease. *The Journal of cell biology*. 2008;183(3):377–84. [PubMed: 18981227]
15. Zhu X, Hill RA, Dietrich D, Komitova M, Suzuki R, Nishiyama A. Age-dependent fate and lineage restriction of single NG2 cells. *Development (Cambridge, England)*. 2011;138(4):745–53.
16. Henry SP, Jang CW, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Generation of aggrecan-CreERT2 knockin mice for inducible Cre activity in adult cartilage. *Genesis (New York, NY : 2000)*. 2009;47(12):805–14.
17. Soriano P Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nature genetics*. 1999;21(1):70–1. [PubMed: 9916792]
18. Ferreira CL, Silva MA, Felicio CM. Signs and symptoms of temporomandibular disorders in women and men. *CoDAS*. 2016;28(1):17–21. [PubMed: 27074184]
19. Nishiyama A, Dahlin KJ, Prince JT, Johnstone SR, Stallcup WB. The primary structure of NG2, a novel membrane-spanning proteoglycan. *The Journal of cell biology*. 1991;114(2):359–71. [PubMed: 1906475]
20. Cameron DA. The Golgi apparatus in bone and cartilage cells. *Clinical orthopaedics and related research*. 1968;58:191–211. [PubMed: 4877096]
21. Kurio N, Saunders C, Bechtold TE, et al. Roles of Ihh signaling in chondroprogenitor function in postnatal condylar cartilage. *Matrix biology : journal of the International Society for Matrix Biology*. 2018;67:15–31. [PubMed: 29447948]
22. Amano K, Densmore M, Nishimura R, Lanske B. Indian hedgehog signaling regulates transcription and expression of collagen type X via Runx2/Smads interactions. *The Journal of biological chemistry*. 2014;289(36):24898–910. [PubMed: 25028519]

Highlights:

1. *NG2-CreER* mouse serves as an additional and powerful Cre-driver to study condylar cartilage.
2. Intraflagellar transport 20 (IFT20) is required for maintaining Golgi size in condylar cartilage.
3. IFT20 is required for ciliary-dependent Hedgehog signaling to produce cartilaginous matrix in condylar cartilage.

Fig. 1.

Evaluation of Cre activity in condylar cartilage.

After crossing *NG2-CreER*, *Col2a1-CreER^{T2}* and *Aggrecan-CreER^{T2}* mice with Rosa26 (R26) reporter mice, tamoxifen was injected intraperitoneally starting from 5 weeks of age.

Condylar cartilage was harvested at 6 weeks and examined. P=Polymorphic layer;

F=Flattened layer; H=Hypertrophic layer.

Fig. 2.

IFT20 is important for condylar cartilage maintenance in mice.

(A) The mandibular condyle was stained with Safranin O at 5 weeks and 3.5 months. (B) PCNA immunohistochemistry and quantifications of PCNA-positive cells at 3.5 months. (C) Total cell number was quantified in the condylar cartilage area at 3.5 months. (D) Cell size distribution in condylar cartilage. (E) Localization of IFT20 and cis-Golgi marker GM130 in condylar cartilage. The size of the Golgi apparatus was quantified. P=Polymorphic layer; F=Flattened layer; H=Hypertrophic layer. Data in (B), (C) and (E) are represented as mean $\pm$ SD; n=3 in each group. * $P<0.05$.

Fig. 3.

IFT20 is essential for ciliary-dependent Hedgehog signaling in condylar cartilage. (A) ATDC5 cells were transfected with scramble and si-*Ift20*, then stimulated with Hedgehog agonist, either Purmorphamine (Puro) or Smoothed agonist (SAG). Immunocytochemistry was performed using anti-acetylated tubulin and anti-Smo antibodies and counterstaining with DAPI. (B) qRT-PCR analysis for *Gli1* and *Ptch1* in control and si-*Ift20* treated ATDC5 cells. (C) Primary cilia were examined by immunohistochemistry of condylar cartilage using anti-acetylated tubulin and anti- γ -tubulin antibodies at 3.5 months. White arrow shows primary cilia in WT. Asterisks indicate the cells shown in the high-magnification image inserted in (C). P=Polymorphic layer; F=Flattened layer; H=Hypertrophic layer. (D) Col X production was examined by immunohistochemistry. P=Polymorphic layer; F=Flattened layer; H=Hypertrophic layer. (E) A mechanism by which IFT20 controls the maintenance of condylar cartilage. IFT20 is critical for governing the Golgi size and regulating Hedgehog signaling to maintain cartilaginous matrix in condylar cartilage. Data in (B) represented as mean $\pm$ SD; n=3 in each group. * P <0.05.