Fibroblast growth factor receptor influences primary cilium length through an interaction with intestinal cell kinase


Abstract

Invertebrate primary cilium is a Hedgehog signaling center but the extent of its involvement in other signaling systems is less well understood. This report delineates a mechanism by which fibroblast growth factor (FGF) controls primary cilium. Employing proteomic approaches to characterize proteins associated with the FGF-receptor, FGR3, we identified the serine/threonine kinase intestinal cell kinase (ICK) as an FGRR3 interactor. ICK is involved in ciliogenesis and participates in control of ciliary length. FGF signalingabolished ICK's kinase activity, through FGR-mediated ICK phosphorylation at conserved residue Tyr15, which interfered with optimal ATP binding. Activation of the FGF signaling pathway affected both primary cilia length and function in a manner consistent with cilia effects caused by inhibition of ICK activity. Moreover, knockdown and knockout of ICK rescued the FGF-mediated effect on cilia. We provide conclusive evidence that FGF signaling controls cilia via interaction with ICK.

Significance

A properly functioning primary cilium is a prerequisite for both normal development and aging of all ciliated organisms, including humans. In vertebrates, the signaling of Hedgehog family morphogens depends entirely on primary cilium. Recently, we reported that fibroblast growth factors (FGF) signaling interacts with that of Hedgehog, and that this is a consequence of FGF regulating length of the cilium and speed of processes that happen therein. In this report, we provide a molecular mechanism of such interaction, identifying intestinal cell kinase as a mediator of the FGF-induced changes in the ciliary morphology and function. This expands our understanding of how FGF signaling regulates intracellular processes, and how aberrant FGF signaling contributes to diseases, such as achondroplasia and cancer.

Keywords

fibroblast growth factor | FGR | intestinal cell kinase | ICK | cilia length


Notes

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To whom correspondence may be addressed. Email: dkrakov@mednet.ucla.edu or krejcip@med.muni.cz.
As the activity of kinases is frequently modulated by transphosphorylation by unrelated kinases, the ciliary kinases represent potential sites of interaction of primary cilia with other signaling systems. In this study, we describe one such mechanism. We unravel how FGF signaling regulates primary cilia length, leading to direct downstream consequences. Using proteomics to characterize the FGFR3 interactome in cells, we identified ICK as an FGFR interactor (17). Here, we demonstrate that FGFRs phosphorylate ICK and partially suppress ICK kinase activity and thus employ ICK to regulate the length and function of primary cilia in cells.

Fig. 1. FGFRs interact with ICK, MAK, and CCRK. (A) IP of FLAG-tagged ICK with V5-tagged wild-type (WT) FGFR3 or activating FGFR3 mutant K650M in 293T cells, or (B and C) FLAG-tagged MAK or CCRK with V5-tagged wild-type FGFR3 or FGFR3-K650M in 293T cells. Actin serves as a loading control. (D) IP of ICK with FGFR1, FGFR2, and FGFR4 demonstrating the ICK association with FGFR1 and FGFR4 but not FGFR2. (E) Wild-type NIH 3T3 cells were transfected with FLAG-tagged ICK together with V5-tagged FGFR3; Ick<sup>Flag</sup> NIH 3T3 cells were transfected only with V5-tagged FGFR3. The antibodies against protein tags were used in the PLA (red); FGFR3 antibody was used to counterstain the transfected cells (green). As a negative control, cells were transfected with FGFR3 and an empty vector (WT), or by GFP (WT and Ick<sup>Flag</sup>). Numbers of PLA dots per cell were calculated and plotted (Student's t test, ***P < 0.001). (Scale bars, 10 μm.) Two clones of Ick<sup>Flag</sup> NIH 3T3 cells, B11, and E5, were analyzed. (F) IP of endogenous FLAG-tagged ICK with endogenous FGFR1 in Ick<sup>Flag</sup> NIH 3T3 cells; actin serves as a loading control. (G–I) Endogenous ICK forms a complex with endogenous FGFR1 in NIH 3T3 cells. (G) Scheme of the procedure, comprising ultracentrifugation, BN-PAGE, SDS/PAGE, and Western blot. (H) Cofractionalization of FGFR1 and ICK-FLAG in Ick<sup>Flag(B11)</sup> NIH 3T3 cells (*). WT NIH 3T3 cells were used as a control. (I) Native complexes (fractions #6 and/or #7) were separated using BN-PAGE, followed by second dimension SDS/PAGE. Orange box shows separation of the ~669-kDa complex containing FGFR1 and ICK-FLAG (arrow).
Results and Discussion

FGFR1, -3, and -4, but Not FGFR2, Interacts with ICK. Tandem mass-spectrometry (MS) was used to identify novel FGFR3 interactors among proteins communoprecipitated (co-IP) with FGFR3 from cells, or among phosphotyrosine proteins isolated from cells with activated FGFR3 signaling. In a total of 26 experiments carried out in 293T cells overexpressing FGFR3, ICK and its homolog male germ cell-associated kinase (MAK) were found in 10 (38%) and 12 (46%) of experiments, respectively (17). Additionally, the ICK-activating kinase, CCRK (18), was identified in 10 (38%) experiments. The ICK association with FGFR3 was confirmed by co-IPs of wild-type FGFR3 and ICK expressed in 293T cells (Fig. 1A). The active FGFR3 mutant K650M that associates with thanatophoric dysplasia (19) also communoprecipitates with ICK. FGFR3 communoprecipitates with MAK and CCRK (Fig. 1 B and C). ICK communoprecipitates with FGFR1 and FGFR4; no association with FGFR2 was found (Fig. 1D).

Because 293T cells do not form cilia, we asked if ICK interacts with FGFR3 in ciliated NIH 3T3 cells. Expressed V5-tagged FGFR3 and FLAG-tagged ICK interacted in intact NIH 3T3 cells by proximity ligation assay (PLA) (Fig. 1E). Next, we used CRISPR/Cas9 to insert FLAG epitope into the Ick locus in NIH 3T3 cells, to generate cells expressing C-terminally 3xFLAG-tagged endogenous ICK (IckFlag cells). PLA showed interaction of endogenous ICK with expressed FGFR3 in two independent IckFlag clones (Fig. 1E). Importantly, IP of endogenous FGFR1 from IckFlag cells demonstrated that endogenous ICK interacts with endogenous FGFR1 (Fig. 1F). In native lysates of IckFlag cells separated at 5–25% sucrose gradients, a cofractionation of FGFR1 with ICK was observed (Fig. 1G and H). Fractions rich in both FGFR1 and ICK were resolved by blue-native (BN)-PAGE to separate protein complexes, which were then analyzed by second-dimension SDS/PAGE to obtain their individual components. Immunoblotting revealed an ~669-kDa protein complex containing FGFR1 and ICK (Fig. 1I).

To further characterize the FGFR–ICK interaction, we generated a series of FGFR3 constructs with truncations in their intracellular domain (Fig. 2A). Mutated variants of FGFR3 were also prepared, by targeting Y724 in the tyrosine kinase (TK) domain, and Y760 and Y770 both located in the C-terminal tail. The Y724/Y760/Y770 mediate interaction with signaling intermediates SH2-βB, p85 PI3K, PLCγ, and GRB14 (20–22). The binding site for the FRS2 adapter was also targeted, either by replacing P418, L419, R425, and V427 with alanines, or by removing the entire region implicated in the interaction (amino acids 406–427) (23). FGFR3 variants were coexpressed with ICK in 293T cells, and analyzed by co-IP. All FGFR3 variants with deleted C terminus did not interact with ICK (Fig. 2B). Similarly, FGFR3-Y724F and -3YF (containing triple substitution Y724F/Y760F/Y770F) did not interact, despite having intact C termini. Deletion of FRS2 binding site had no effect on FGFR3 interaction with ICK, similar to Y760F or Y770F substitutions, which communoprecipitated with ICK normally.

Fig. 2 shows that both the C terminus of FGFR3 and Y724 are required for ICK binding. To characterize in detail the FGFR3 epitopes involved in interaction with ICK, a peptide microarray technology was used. Short peptides (7–22 aa)
covering the intracellular part of FGFR3 were synthesized and immobilized on a glass slide, incubated with recombinant ICK, and the peptide–ICK interaction was analyzed as detailed in Materials and Methods. Strong ICK binding was obtained for the FGFR3 peptide $748^{\text{TFKQLVEDLDRVLTSTDEY}}$760, located at the boundary between the TK domain and the C term (SI Appendix, Fig. S4). Interestingly, shorter versions of this peptide, specifically $748^{\text{DRVLTSTDEY}}$760 and $748^{\text{TFKQLVEDL}}$, did not show any ICK binding (Fig. S4 and SI Appendix, Fig. S1B). Crystal structure of the FGFR3 TK domain (PDB ID code 4K33) shows that $748^{\text{TFKQLVEDL}}$ forms an $\alpha$-helix followed by an intrinsically disordered $754^{\text{TFKQLVEDL}}$ sequence (Fig. S4B). It is thus possible that ICK binds only to a correctly assembled secondary structure in FGFR3, and not to the peptides lacking either the helical or the unstructured parts of the $748^{\text{TFKQLVEDL}}$ motif. This is supported by the IP data, where the C-lobe of the TK domain alone or the C terminus alone did not interact with ICK (Fig. 2B) (FGFR3-ΔC-t and FGFR3-ΔTK). Next, we asked whether differences in the sequence of FGFR3 motif involved in ICK interaction could account for the lack of FGFR2 association with ICK, observed in co-IP experiments (Fig. 1D). We replaced the $751^{\text{VLTSTDEY}}$ in FGFR3 with homologous FGFR2 sequence $760^{\text{ILTLTTNEEY}}$, and determined the interaction of chimeric FGFR3 (FGFR3-R2-C-t) with ICK using co-IP. Fig. 3C demonstrates that FGFR3-R2-C-t capacity to co-IP with ICK diminished by $\sim 40\%$, compared with the wild-type FGFR3.

Our data indicate that the Y724 and C terminus of the FGFR3 are both essential for ICK binding: FGFR3-Y724F has an intact C terminus but does not bind ICK. Similarly, the FGFR3 constructs with a deleted C terminus did not bind ICK, despite having the Y724 intact (Fig. 2B). Thus, the ICK binding to either site is rather weak and cooperativity between these two sites is required in the context of the 3D FGFR3 structure (Fig. 3B). Alternatively, the Y724 mediates ICK binding indirectly, acting as an allosteric element controlling accessibility of the C terminus for ICK.

**FGF Signaling Triggers Cytoplasmic Accumulation of ICK.** The association of wild-type FGFR3 and FGFR3-K650M with ICK was confirmed by PLA, carried out with 293T cells expressing V5-tagged FGFR3 and FLAG-tagged ICK (Fig. 4A). Immunocytochemistry revealed an overlap of FGFR3 signal with ICK in the cytoplasm, which contrasted with predominant nuclear ICK localization in cells not expressing FGFR3 (Fig. 4B), suggesting that FGFRs could cause ICK’s retention in the cytoplasm. We tested this hypothesis by determining the ICK localization in 293T cells, where the signaling of endogenous FGFR1–4 (24) was activated by addition of FGFR ligand FG2. Progressive cytoplasmic accumulation of ICK was found in cells treated with FGFR3 (Fig. 4 C and D). Expression of active FGFR3-K650E or -K650M also retained ICK in the cytoplasm (Fig. 4E). Thus, the activation of FGF signaling alters subcellular localization of ICK, causing its cytoplasmic accumulation.

**FGFRs Phosphorylate ICK and Inhibit ICK Kinase Activity.** ICK shuttles between the cytoplasm and nucleus (25) and this is affected by kinase activity, as demonstrated by cytoplasmic accumulation of the partially inactive ICK mutant R272Q (26). Because FGF signaling altered ICK subcellular localization (Fig. 1C), we asked whether it affected ICK activity through phosphorylation. Kinase assays utilizing recombinant FGFR3 and ICK revealed that FGFR3 phosphorylated ICK at tyrosine residues (Fig. 5A, lane 4). Kinase assays carried out with recombinant MAK and CCRK yielded similar results (Fig. 5 B and C, lane 4). Phosphoryrosine mapping identified several ICK tyrosines phosphorylated by FGFR3, among which Y15, Y156, Y495, and Y555 are conserved in ICK and MAK in human, mouse, chick, *Xenopus*, and zebrafish (Fig. 5D). Y495 and Y555 localized to an unstructured regulatory region of ICK (amino acids 320–632 for human ICK), making it impossible to predict the effect of their phosphorylation on ICK function. In contrast, the Y15 lies within the highly structured ICK kinase domain. In silico modeling revealed that phosphorylation at Y15 positions a negatively charged phosphate moiety in immediate proximity to the pocket used for binding of an ATP phosphate group, suggesting that phosphorylation at Y15 down-regulates ICK kinase activity via interference with optimal ATP binding (Fig. 5D). We tested this prediction by determining the kinase activity of ICK, immunopurified from 293T cells in which endogenous FGFR was activated by treatment

![Fig. 3. The $751^{\text{VLTSTDEY}}$ motif in FGFR3 is required for the interaction with ICK. (A) Averaged fluorescence intensities from three replicates of the peptide microarray involving peptides from FGFR3 C-terminal region. (B) Ribbon and surface representations of the crystal structure of the TK domain of FGFR3 (PDB ID code 4K33). Residue Y724 and C-terminal region implicated in ICK binding by co-IP experiments and peptide microarray analysis are highlighted in blue and orange/green, respectively. Orange, $\alpha$-helix; green, the residue $751^{\text{VLTSTDEY}}$ (unstructured). Note that the absence of structural information for residues $760^{\text{TFKQLVEDL}}$ is suggestive of structural disorder. (C) The putative ICK interacting motif on FGFR3 ($751^{\text{VLTSTDEY}}$) was replaced by the analogous sequence from FGFR2 ($760^{\text{ILTLTTNEEY}}$), creating the FGFR3-R2-C-t chimera. The co-IP of FGFR3-R2-C-t with ICK compared with wild-type FGFR3 (Student’s $t$ test; ***$P < 0.001$).](image-url)
with FGF2. FGF2 induced accumulation of expressed ICK, but its kinase activity diminished by ~30% at the same time, as determined in a kinase assay utilizing myelin basic protein (MBP) as a substrate and P<ATP to visualize ICK phosphorylation (Fig. 5E). Based on FGF treatment producing accumulated cytoplasmic ICK, it predicts that the degree of ICK accumulation should induce MBP phosphorylation at commensurate levels. However, the results show little induction of MBP phosphorylation after FGF2 treatment, suggesting partial inhibition of ICK activity by FGF (Fig. 5E). Because the MBP kinase assay is a cell-free experiment, we tested whether the FGF signaling inhibited ICK activity in cells. In 293T cells coexpressing ICK and mammalian target of rapamycin (mTOR) complex 1 protein Raptor (mTOR), the levels of ICK kinase activity were determined by detecting previously established ICK-mediated Raptor phosphorylation at T908 (27). Treatment with FGF2 caused accumulation of ICK, but the pRaptor(T908) levels increased only weakly, corresponding to 51–55% inhibition of relative ICK activity (Fig. 5F). These experiments demonstrate that interaction with FGFRs stabilizes cytoplasmic ICK while partially downregulating its kinase activity.

**FGF Signaling Regulates the Cilia Length via ICK.** Because both ICK and FGF regulate cilia length (4, 15), we asked whether FGF signaling regulates cilia via ICK. NIH 3T3 cells were serum-starved for 12 h to produce primary cilia, treated with FGF2 for up to 24 h, and cilia were visualized by immunostainings for the axoneme (acetylated tubulin), ciliary membrane (ARL13B), and centrioles (pericentrin). FGF2 triggered progressive increase in cilia length peaking at 12 h (3.13 ± 0.05 μm vs. 2.33 ± 0.04 μm in controls) (Fig. 6A). Transfection of two independent Ick shRNAs resulted in ~20–40% knockdown of Ick expression in NIH 3T3 cells, with corresponding (11–18%) extension of primary cilia length, compared with nontransfected controls or cells transfected with scrambled shRNA (Fig. 6B). Importantly, the cilia in Ick shRNA cells were resistant to FGF2-mediated elongation, in contrast to scramble shRNA or control cells, which responded to FGF2 with cilia elongation (31–33%). Next, we down-regulated ICK in Ick(Δ388H11) NIH 3T3 cells by stable transfection of doxycycline (DOX)-inducible lentiviral shRNA construct. DOX caused ~67% down-regulation of the ICK protein. DOX-induced cells were resistant to FGF2-mediated cilia elongation, in contrast to controls, which responded to FGF2 with cilia elongation (Fig. 6C). Finally, we tested whether chemical inhibition of ICK kinase activity affects cilia length. According to the DrugKiNET database (www.drugkINET.ca), flavopiridol, AT7519, and lestaurotinib act as chemical ICK inhibitors, with KD values of 0.69 nM, 8.3 nM, and 39 nM, respectively (28). NIH 3T3 cells treated with flavopiridol, AT7519 or lestaurotinib showed concentration-dependent deregulation of cilia length. Importantly, FGF2 failed to elongate these cilia (SI Appendix, Fig. S2).

Next, the Ick locus in NIH 3T3 cells was inactivated by CRISPR/Cas9. A sequence corresponding to Glu80 in ICK was targeted, because it localizes to the ATP binding pocket and its substitution with Lys abolishes ICK kinase activity (16). We failed to generate IckE80K cells, but nevertheless produced clones with disrupted Ick. Interestingly, none of the 302 clones obtained in three rounds of CRISPR targeting possessed complete inactivation of all three Ick loci in triploid NIH 3T3 cells (29), suggesting that some level of ICK protein is essential for NIH 3T3 growth. Three clones were selected for further analyses, in which the two Ick alleles were inactivated, and the remaining one allele contained in frame deletions ranging from 3 to 14 residues surrounding the Glu80 (Fig. 7A). Ick mRNA was down-regulated to about 40–60% among the selected clones, but the protein levels remained unchanged (Fig. 7B and C), again suggesting that presence of the ICK protein is necessary for NIH 3T3 survival. The ICK deletions generated by CRISPR were introduced into the wild-type ICK via site-directed mutagenesis, and the resulting ICK variants were evaluated for kinase

**Fig. 4.** FGF signaling alters ICK’s subcellular distribution. (A) 293T cells were transfected with FLAG-tagged ICK together with VS-tagged wild-type FGFR3 or its active mutant K650M. The antibodies against protein tags were used in the PLA (red); FGFR3 antibody was used to counterstain the transfected cells (green). As a negative control, cells were transfected with FGFR3 and an empty vector. Numbers of PLA dots per cell were calculated and plotted (Student’s t test, ***P < 0.001). (Scale bar, 10 μm.) (B) Increased cytosolic localization of transfected ICK in a 293T cell cotransfected with FGFR3-K650E, determined by ICK and FGFR3 immunocytochemistry (Scale bar, 20 μm.). (C and D) Altered ICK subcellular distribution in 293T cells expressing FLAG-tagged ICK, treated with FGF2; ICK was visualized by FLAG immunocytochemistry. (C) Typical localization patterns of ICK (DIC, differential interference contrast). (Scale bar, 20 μm.) (D) Percentages of cells in each category of ICK localization (Student’s t test, ***P < 0.01). (E) 293T cells were transfected with FLAG-tagged wild-type FGFR3, active FGFR3-K650E, or K650M, or empty vector, and immunoblotted for phosphorylated (p) FGFR3. ICK and FGFR3 were visualized by immunocytochemistry, and ICK subcellular localization was determined.
activity in 293T cells. We observed a dramatic reduction in ICK activating autophosphorylation at the Y159 (25) in all three CRISPR variants, compared with wild-type ICK, demonstrating that IckCRISPR cells express normal levels of kinase-dead ICK (Fig. 7D).

Severe abnormalities were found in the cilia of IckCRISPR cells, which we termed a “cilia disaster” phenotype. These cilia were highly variable in length (0.4–6.9 μm; CV 65.14%; n = 296), compared with relatively narrow range in control cells (1.2–4.4 μm; 22.05%; n = 409) (SI Appendix, Fig. S3). The IckCRISPR cilia displayed abnormal morphology, often manifested as rudiments negative for acetylated and polyglutamylated tubulin (Fig. 7E, Bottom). Long and twisted cilia in some IckCRISPR cells also appeared less stable than wild-type cilia, as suggested by lesser stability of ICK immunoprecipitated from FGF2-treated 293T cells, measured using MBP as a substrate, in the presence of P32-P-ATP. ICK activity is presented as a relative %MBP/MBP ratio (“Obtained”), and compared with the expected values based on ICK amounts entering the kinase assay quantified using Coomassie stained gel (ICK Coomassie) or ICK immunoblot (ICK IP) (Student’s t test, ***P < 0.001). The percentages express the extent of inhibition of the ICK kinase activity in FGF2-treated cells.

We next evaluated the effect of FGF2 on cilia length in fibroblasts established from an individual with lethal short rib polydactyly syndrome due to an inactivating E80K mutation in ICK (16). Compared with control fibroblasts, ICK-E80K cells exhibited greater range in their cilia length distribution, with many long and twisted cilia (Fig. 7F). Treatment with FGF2 caused statistically significant cilia elongation in control fibroblasts but not in ICK-E80K cells.

Finally, the limb bud micromass cultures were used to evaluate the FGF2 effect on cilia. FGF2 caused primary cilia extension in micromasses established from embryonic day 12 (E12) mouse limb buds isolated from wild-type or Ick−/− mice (Fig. 7F) (4). Importantly, this effect depended on ICK, as cells derived from Ick−/− mouse limb buds (13) were insensitive to FGF2-mediated
Fig. 6. FGF regulates the length of primary cilia via ICK. (A) Primary cilia length extension in NIH 3T3 cells treated with FGF2. Cilia were visualized by ARL13B, acetylated tubulin (AcTu) and pericentrin immunostaining, measured in 3D and plotted. Black dots, individual cilia; red bars, medians. (Scale bars: 5 μm (cells) and 1 μm (cilia).) (B) Rescue of FGF2-mediated cilia extension with two independent Ick shRNAs (Ick #1 and Ick #2). Ick transcript levels were monitored by qPCR at 24 h (beginning of serum starvation) and 36 h (FGF2 treatment) after transfection, and normalized to Gapdh expression. The columns show Ick expression levels relative to the scrambled control (red dashed line). Cilia length was measured 48 h after transfection and graphed. (C) Rescue of FGF2-mediated cilia extension in Ick−/− NIH 3T3 cells stably transfected with DOX-inducible shRNA-expressing construct targeting Ick expression. Ick protein levels were monitored by immunoblot after 4 d with DOX (beginning of FGF2 treatment), normalized to actin, and plotted. Cells expressing scrambled (Scr.) shRNA upon DOX were used as a control. Student's t test, **P < 0.01, ***P < 0.001; n.s., not significant.

Regulation of Primary Cilia by FGF–ICK Pathway. The regulation of primary cilia length and function by FGF signaling has recently emerged as a new paradigm in cell biology (3–5). However, the molecular mechanism by which FGFRs regulate cilia remains unclear. In this article, we uncover that FGFRs interact with and phosphorylate an important and conserved ciliary kinase, ICK, leading to partial inhibition of its kinase activity and altered subcellular localization (Figs. 1–5). Modulations of FGF signaling regulate primary cilia length, IFT velocity, and Hh signaling consistent with effects caused by its inhibition of ICK activity (3, 4, 15, 16, 33). Moreover, ICK removal or down-regulation abolished the FGF-mediated effect on ciliary length (Figs. 6 and 7), demonstrating that FGF signaling regulates primary cilia via ICK. In the following section, we describe known cilia phenotypes regulated by FGF signaling, and explain these phenotypes on the basis of the FGF–ICK interaction (Fig. 8).

First, the ICK is a sensitive regulator of primary cilia length. Deletion of ICK homologs LF4, LmxMPK9, and DYT-5 increased the length of cilia in green algae (Chlamydomonas reinhardtii), protists (Leishmania mexicana), and worms (Caenorhabditis elegans) (10–12). Similarly, Ick−/− mice or humans carrying partially or completely inactivating ICK mutations R272Q and E80K showed elongated primary cilia (13, 16, 34). Expression of these variants and other inactive ICK mutants in cultured cells, or partial down-regulation of Ick expression via RNA interference also elongated primary cilia (13–16, 35). Because FGFRs phosphorylate and inactivate ICK (Fig. 5), a down-regulation of FGF signaling should relieve this inhibition, resulting in elevated ICK activity and shorter cilia (Fig. 8B). Indeed, mice treated with chemical inhibitors of FGFR activity showed shorter cilia in the biliary ducts, kidney, and lung (4). This is corresponding to the evidence obtained in zebrafish and Xenopus, where inactivation of Fgf signaling led to shorter cilia in multiple tissues (3). Similar to ICK inactivation, stimulation of FGF signaling elongated primary cilia in NIH 3T3 cells, primary mouse embryonic and human fibroblasts, epithelial IMCD3 cells, and mouse limb bud mesenchymal cells (4), but failed to do so in Ick−/− background or in cells endogenously expressing inactive ICK (Fig. 7). Similarly, cells with diminished Ick expression or activity were insensitive to the FGF-mediated cilia elongation (Fig. 6), demonstrating that FGF signaling elongates primary cilia via inhibition of ICK kinase activity (Fig. 8C).

While inhibition of ICK activity extended primary cilia, experimental up-regulation of ICK activity produced shorter cilia (11–13, 16, 33). Interestingly, ICK inactivation also led to cilia shortening in at least two instances, in the neural tube and embryonic fibroblasts isolated from an Ick−/− mice (33), and in the NIH 3T3 IckCRISPR cells reported here (Fig. 7A–G and SI Appendix, Fig. S3) (the cilia disaster phenotype). In our previous work, we showed that FGF signaling can also shorten primary cilia (4, 5), as observed in cells expressing constitutively active FGFR3-K650E or -K650M mutants. Interestingly, these FGFR3 mutants interacted with ICK (Fig. 1). Thus, the FGF-mediated ICK inactivation may lead to profound dysregulation of ciliogenesis manifested as cilia shortening or extension, depending on the strength and duration of the FGF stimulus (Fig. 8D).

Ick−/− mouse models described by both Chaya et al. (33) and Moon et al. (13) displayed preaxial polysyndactyly and dwarfism, a similar phenotype to human short-rib polydactyly syndrome associated with an inactivating ICK mutation (16), or to mice and human with endocrine-cerebro-osteodysplasia syndrome coupled with mildly inactivating ICK mutations (26, 35, 36). These phenotypes suggest impaired Hh signaling, as polydactyly stems from defective Hh function in early limb patterning (37), while the shortened appendicular skeletons likely result from disrupted...
FGF signaling causes ICK retention in the cytoplasm. This suggests that FGFRs may alter intracellular distribution of ICK, thus impeding ICK cilia function by its sequestration away from cilia, in addition to partial inactivation. Second, despite numerous reports describing an essential role of ICK in maintenance of proper cilia form and function, the molecular mechanism of this phenotype is poorly known.

Materials and Methods

Cell Culture, Vectors, Transfection, RNAi, CRISPR/Cas9, Lentiviruses, and qPCR

The 293T and NIH 3T3 cells were obtained from ATCC. Shh-LIGHT2 cells were a kind gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an internal gift from P. Beachy, Stanford University School of Medicine, Stanford, CA (32).

FGFR1, and FGFR3 are known to localize to the cilia (5, 13–16, 33, 35, 39, 40), it is possible that they interact in the cilium or in its basal body. Alternatively, the FGFRs may interact with ICK outside of the cilium. We and others (16, 25) show that the majority of the ICK protein shuttles between nucleus and cytosol, and activation of FGF signaling causes ICK retention in the cytoplasm. This suggests that FGFRs may alter intracellular distribution of ICK, thus impeding ICK cilia function by its sequestration away from cilia, in addition to partial inactivation. Second, despite numerous reports describing an essential role of ICK in maintenance of proper cilia form and function, the molecular mechanism of this phenotype is poorly known. ICK phosphorylates Kif3a (13), but how this phosphorylation affects Kif3a function or IFT velocity is not known. Further understanding of the FGF–ICK regulation of cilia will provide insights into the pathology of Mendelian inherited disorders and cancers caused by defective FGF signaling.
Gradient Ultrafiltration, BN-PAGE, Western Blot, IP, Kinase Assays, and Peptide Microarrays. Native cell lysates (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP40, 0.5% Igepal CA-630, 1 mM EDTA pH 8.0, 0.25% sodium deoxycholate, 1 mM Na3VO4, proteinase inhibitors) were cleared, loaded on 5–25% sucrose gradient (1 mM Tris-HCl pH 7.4, 150 mM NaCl, 3 mM MgCl2, proteinase inhibitors), and centrifuged at 40,000 rpm/°C/16 h using SW 50 Ti swinging bucket rotor (Beckman Coulter). Gradient fractions were either precipitated with 10% TCA or used for BN-PAGE. For BN-PAGE, fractions were mixed with solubilization buffer (50 mM NaCl, 50 mM imidazole-HCl, 1 mM EDTA, pH 7) and concentrated using Spin-X UF 500 (cut-off 30 kDa; Corning). Before sample loading, the wells of the 4–15% native tricine–imidazole gel were washed with cathode buffer (0.5 mM tricine, 7.5 mM imidazole, 0.02% Coomassie blue G250, pH 7–7), and the gel run at constant 15 mA/°C. The 29- to 66-kDa native protein ladder was from Sigma. Individual lanes were cut from the gel, placed over the SDS gel, and protein complexes were separated using second dimension SDS/PAGE, and analyzed by Western blot. For Western blot, lysates were resolved by SDS/PAGE, transferred onto a PVDF membrane and visualized by chemiluminescence (Thermo). The following antibodies were used: GADPH (S1574), GL11 (2643), FGFR1 (9740), actin (3700; Cell Signaling); HA (sc-0853), FGFR3 (sc-123), pFGFR3 Y737/924 (sc-33041; Santa Cruz Biotechnology); FLAG (F1804), GST (G1160; Sigma); VS (R960-25; Invitrogen), 4G10 (05-321; Millipore), pIC8Kβ (ab138435; Abcam), GL3 (Abcam); pRaptor (Addgene); and pICK (sc-27). For IP, proteins were extracted in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 2 mM EDTA pH 8.0, 0.5 mM DTT, proteinase inhibitors; immunocomplexes were collected on protein A/g agarose (Santa Cruz). Kinase assays were performed using recombinant FGFR3 together with ICK, MAK, or CCRK (SignalChem), in kinase buffer (60 mM Hepes pH 7.5, 3 mM MgCl2, 3 mM MnCl2, 3 μM Na3VO4, 1.2 mM DTT) in the presence of 10 μM Ick substrate phosphorylation. Triplicates were obtained by Western blot with 4G10 antibody (Millipore). [γ-32P]-ATP kinase assays were carried out with IP ICK and 4 μg of recombinant MBP (Sigma) as a substrate, in a kinase buffer (50 mM Hepes pH 7.5, 10 mM MnCl2, 10 mM MgCl2, 8 μM β-glycerophosphate, 1 mM DTT, 0.1 mM Na3VO4, 0.1 mM PMSF), in the presence of 1 μCi [γ-32P]-ATP (Izotop). Samples were resolved by SDS/PAGE and visualized by autoradiography. Band intensities were quantified in ImageJ. Peptide libraries corresponded to primary sequence of FGFR3 intracellular arms (47). These proteins were synthesized as N-terminally tagged peptides on a glass slide via hydrophilic linker (Peptstar microarray; JPT Peptide Technologies). The library contained overlapping peptides (13-aa long with 10-aa overlap) covering disordered parts of FGFR3 in the juxtamembrane and C-terminal regions, and nonoverlapping peptides of varying length that emulated elements of the secondary structure exposed on the surface of FGFR3 TK domain (FGFR3: 4K33) (SI Appendix, Fig. S1). Microarrays were incubated with recombinant ICK (SignalChem), in kinase buffer containing 10 μM of recombinant phosphopeptide, which was then purified by Western blot and used for BN-PAGE and visualized by autoradiography. The 3D image was obtained by Berkeley Microarray Scanner 4000 SL50 and quantified using GenePix (Molecular Devices). The peptides with fluorescence intensity at least 10-fold above nonspecific were considered as potential binding sites. Microarray preparation, data acquisition, and analysis were described previously (47).

Immunocytochemistry, PLA, and Cilia Length Measurements. Cells were fixed in paraformaldehyde and incubated with the following antibodies: VS (R960-25), acetylated α-tubulin (23-2700; Invitrogen), FLAG (F1804; Sigma), ARL13B (17711-1-AP; Proteintech), γ-tubulin (ab11316), pericentrin (ab4448; Abcam), polyglutamylated tubulin (AG-20B-0020-C100; AdipoGen), SMO (sc-25), acetylated α-tubulin (ab11316), and γ-tubulin (ab4448; Abcam). Cells were fixed in 4% paraformaldehyde and incubated with the following antibodies: vs-FLAG (ab11316; Abcam), 4G10 (ab138435; Abcam), GL3 (Abcam); pRaptor (Addgene); and pICK (sc-27). For IP, proteins were extracted in buffer containing 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.1% sodium deoxycholate, 2 mM EDTA pH 8.0, 0.5 mM DTT, proteinase inhibitors; immunocomplexes were collected on protein A/g agarose (Santa Cruz). Kinase assays were performed using recombinant FGFR3 together with ICK, MAK, or CCRK (SignalChem), in kinase buffer (60 mM Hepes pH 7.5, 3 mM MgCl2, 3 mM MnCl2, 3 μM Na3VO4, 1.2 mM DTT) in the presence of 10 μM Ick substrate phosphorylation. Triplicates were obtained by Western blot with 4G10 antibody (Millipore). [γ-32P]-ATP kinase assays were carried out with IP ICK and 4 μg of recombinant MBP (Sigma) as a substrate, in a kinase buffer (50 mM Hepes pH 7.5, 10 mM MnCl2, 10 mM MgCl2, 8 μM β-glycerophosphate, 1 mM DTT, 0.1 mM Na3VO4, 0.1 mM PMSF), in the presence of 1 μCi [γ-32P]-ATP (Izotop). Samples were resolved by SDS/PAGE and visualized by autoradiography. Band intensities were quantified in ImageJ. Peptide libraries corresponded to primary sequence of FGFR3 intracellular arms (47). These proteins were synthesized as N-terminally tagged peptides on a glass slide via hydrophilic linker (Peptstar microarray; JPT Peptide Technologies). The library contained overlapping peptides (13-aa long with 10-aa overlap) covering disordered parts of FGFR3 in the juxtamembrane and C-terminal regions, and nonoverlapping peptides of varying length that emulated elements of the secondary structure exposed on the surface of FGFR3 TK domain (FGFR3: 4K33) (SI Appendix, Fig. S1). Microarrays were incubated with recombinant ICK (SignalChem), in kinase buffer containing 10 μM of recombinant phosphopeptide, which was then purified by Western blot and used for BN-PAGE and visualized by autoradiography. The 3D image was obtained by Berkeley Microarray Scanner 4000 SL50 and quantified using GenePix (Molecular Devices). The peptides with fluorescence intensity at least 10-fold above nonspecific were considered as potential binding sites. Microarray preparation, data acquisition, and analysis were described previously (47).

MS, Modeling, Animal Experiments, Immunohistochemistry, and Statistics. Kinase reactions containing FGFR3 and ICK were subjected to reduction, alkyla- tion, and in-solution digestion by trypsin. Samples were analyzed using nanoscale liquid chromatography connected to the tandem mass spec- trometer (RSCLnano connected to Orbitrap Elite; Thermo Fisher). High-resolution HCD or ETD MS/MS spectra were acquired in the Orbitrap analyzer. The analysis of the MS RAW data files was carried out using the Protein Discoverer software (v.1.4; Thermo) with a Mascot search engine. Quantitative information assessment and phos- phopeptide signal validation was done in Skyline. The 3D ICK model was obtained via template-based modeling using the PHyre software (48). The ICK-specific functional elements, predicted using the National
Center for Biotechnology Information Conserved Domain Database (49), were mapped onto a 3D model of the ICK using the CHIMERA software (50). Moreover, homology models (CASP11) were constructed to dock AICD into the ICK ATP binding site. Ick-ΔC mice were described previously (16). Ick−/− mice were maintained in a C57BL/6N background and used as controls. For micromasses, the primary mesenchymal cells were harvested from the limb buds of E12 mouse embryos, digested with dispase II, and spotted in 10-μL aliquots at 2 × 10^5 cells/mL. Cells were allowed to adhere before differentiating media (50% F12/40% DMEM, 10% FBS, 50 μg/mL ascorbic acid, 10 mM β-mercaptoethanol, 50 μM β-D-glucuronide) was added for 1 d in media supplemented with FGF2 (Sigma), fixed with methanol and immunolabeled by ARL13B and γ-tubulin (6557; Sigma) antibodies. For GL2 detection, micromasses were serum-starved for 12 h, treated with FGF2 and SAG for additional 12 h, and immunostained using GL2 and ARL13B antibodies (13). Animal experiments were approved by the Institutional Animal Care and Use Committee of Dongguk University, Korea (IACUC-2016-016-1). All experiments were performed at least in triplicate unless stated otherwise. The n values express the number of independent biological experiments. Data are presented as mean ± SEM. Two-tailed Student’s t test was used for statistical analysis of data. Brightness and contrast were adjusted in microphotographs, homogeneously throughout each panel.

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