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

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Vertebrate 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, FGFR3, we identified the serine/threonine kinase intestinal cell kinase (ICK) as an FGFR interactor. ICK is involved in ciliogenesis and participates in control of ciliary length. FGF signaling partially abolished ICK’s kinase activity, through FGFR-mediated ICK phosphorylation at conserved residue Tyr15, which interfered with optimal ATP binding. Activation of the FGF signaling pathway affected both primary cilium length and function in a manner consistent with cilium effects caused by inhibition of ICK activity. Moreover, knockdown and knockout of ICK rescued the FGF-mediated effect on cilium. We provide conclusive evidence that FGF signaling controls cilia via interaction with ICK.

* Fibroblast growth factor | FGFR | Intestinal cell kinase | ICK | Cilia length

In vertebrates, the signaling of Hedgehog (Hh) morphogens depends entirely on primary cilium. The cilium provides a structural and functional compartment that integrates a series of intricate molecular mechanisms allowing cells to process Hh-target transcriptional regulators and alter gene-expression programs in response to Hh (1). Giving the rapidly growing importance of primary cilium in the regulation of physiologic and pathologic cellular functions (2), many other signaling systems are anticipated to work through the cilium.

Several recent lines of evidence demonstrate that fibroblast growth factors (FGF) regulate primary cilium. Inactivation of the FGF-receptor Fgfr1 or its FGF ligand lead to shorter cilia in zebrafish and Xenopus (3). In mammals, FGF signaling regulates the length of primary cilium in skin fibroblasts, lung, kidney, and liver cells, human embryonic stem cells and human induced pluripotent stem cells, embryonal fibroblasts, and mesenchymal cells (4). In addition, the human skeletal dysplasias caused by activating FGFR3 mutations, such as achondroplasia, manifest by abnormal cilia (4, 5). Evidence strongly suggests that FGF signaling integrates cilia into the canonical FGF signaling pathway. However, the mechanism through which FGFR’s regulate primary cilium is not known.

Several serine/threonine kinases control ciliogenesis or other specific functions of primary cilia. These “ciliary kinases” include TTBK2 and GSK3β, involved in initiation of ciliogenesis and assembly of the ciliary membrane (6, 7), NEK2, which regulates cilia disassembly (8), and CK1 and GRK2, which are important for Smoothened (SMO) translocation into the cilia (9). The MAP-kinase superfamily kinase intestinal cell kinase (ICK) is another well-known regulator of primary cilium, conserved in this function from single-celled organisms to mammals. Deletion of ICK or its homologs increases the cilia length in green algae, protists, and nematodes in vivo (10–12). In cultured mammalian cells, down-regulations of ICK kinase activity lead to extended and abnormal cilia, demonstrating that ICK is an essential regulator of the length of primary cilia (13–16).

**Significance**

A properly functioning primary cilium is 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 how FGF signaling regulates intracellular processes, and how aberrant FGF signaling contributes to diseases, such as achondroplasia and cancer.

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The authors declare no conflict of interest.

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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>−/−</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>−/−</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>−/−</sup> NIH 3T3 cells, B11, and E5, were analyzed. (F) IP of endogenous ICK with endogenous FGFR1 in Ick<sup>−/−</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>−/−</sup>B11 NIH 3T3 cells (*). WT NIH 3T3 cells were used as a control. (I) Native complexes (fractions #6 and #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 coimmunoprecipitated (co-IP) with FGFR3 from cells, or among phosphorytrosine 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 coimmunoprecipitates with ICK. FGFR3 coinmunoprecipitates with MAK and CCRK (Fig. 1B and C). ICK coimmunoprecipitates 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 alamines, 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 coimmunoprecipitated 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 TFKQVELDRVLTSTDEY\textsuperscript{750} located at the boundary between the TK domain and the C terminus (\textit{SI Appendix, Fig. S1A}). Interestingly, shorter versions of this peptide, specifically LDRVLTSTDEY\textsuperscript{750} and TFKQVELDRVLTV\textsuperscript{754}, did not show any ICK binding (\textit{SI Appendix, Fig. S1B}). Crystal structure of the FGFR3 TK domain (PDB ID code 4K33) shows that TFKQVELDRVLTV\textsuperscript{754} forms an α-helix followed by an intrinsically disordered VLTSTDEY\textsuperscript{750} sequence (Fig. 2B). 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 TFKQVELDRVLTVSTDEY\textsuperscript{760} 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 LDRVLTSTDEY\textsuperscript{760} in FGFR3 with homologous FGFR2 sequence ILTLTTINEE\textsuperscript{760}, 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 ~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 cooperative between these two sites is required in the context of the 3D FGFR3 structure (Fig. 2B). 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 FGFR2 (Fig. 4C 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. 4), 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. 5B and C, lane 4). Phosphotyrosine 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, \textit{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 with FGF2. Progressive cytoplasmic accumulation of ICK was found in cells treated with FGFR2 (Fig. 4C 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.
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 consensurate 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, 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 FGFRs 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 short-hairpin (sh)RNAs 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\(_{\text{lsat}}\) 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 lestaurtinib act as chemical ICK inhibitors, with \(K_D\) values of 0.69 nM, 8.3 nM, and 39 nM, respectively (28). NIH 3T3 cells treated with flavopiridol, AT7519 or lestaurtinib 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 Glu60 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 Ick\(_{\text{E60K}}\) 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 Glu60 (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
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 Ick<sup>CRISPR</sup> cells express normal levels of kinase-dead ICK (Fig. 7D).

Severe abnormalities were found in the cilia of Ick<sup>CRISPR</sup> 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 Ick<sup>CRISPR</sup> cilia displayed abnormal morphology, often manifested as rudiments negative for acetylated and polyglutamylated tubulin (Fig. 7E, Bottom). Long and twisted cilia in some Ick<sup>CRISPR</sup> cells also appeared less stable than wild-type cilia, as suggested by lesser axoneme staining for acetylated tubulin (30) (Fig. 7E, arrow). Ick<sup>CRISPR</sup> cells showed profound disruption of Hh signaling, manifested as failure to process the GLI3 transcriptional regulator in response to Hh agonist SAG (SMO agonist) (31), and to induce expression of Hh target genes Gli1 and Ptc1 (SI Appendix, Fig. S4 A–C). Mislocalization of GLI3 and the components of the cilia transport BBS8 and intraflagellar transport (IFT)172 was found in the Ick<sup>CRISPR</sup> cilia tips (SI Appendix, Fig. S4D). Finally, the Hh coreceptor SMO localized into the cilia in SAG-naïve Ick<sup>CRISPR</sup> cells, in contrast to wild-type controls, which localized SMO to cilia only when treated with SAG (SI Appendix, Fig. S4E), suggesting increased permeability in Ick<sup>CRISPR</sup> cilia.

FGF2 did not elongate cilia in any of the three Ick<sup>CRISPR</sup> clones; however, the informative value of this data may be affected by the profound ciliary dysregulation (Fig. 7F). Addition of wild-type ICK into the Ick<sup>CRISPR</sup> background partially reversed the cilia disaster phenotype, resulting in formation of many cilia of normal length, which responded to FGF2 with usual elongation (Fig. 7G). Addition of kinase-dead ICK-E80K also rescued the cilia disaster; however, these cilia were longer than those in wild-type ICK add-back cells and, importantly, were resistant to FGF2-mediated elongation (Fig. 7G).

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. 7H). 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<sup>−/−</sup> mice (Fig. 7I) (4). Importantly, this effect depended on ICK, as cells derived from Ick<sup>−/−</sup> mouse limb buds (13) were insensitive to FGF2-mediated
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 FGFs 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 Fgfl 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 Ick\(^{-/-}\) cells reported here (Fig. 7 A–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 polydactyly and dwarfism, a similar phenotype to human short-rib polydactyly syndrome associated with an inactivating ICK mutation (16), or to mice and humans 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

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**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. (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 bars, medians). (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.

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...cilia elongation. Taken together, the ICK deletion or down-regulation of ICK kinase activity rescued the FGF-mediated elongation of primary cilia. These findings establish that FGF signaling regulates cilia length via ICK.

Because ICK inactivation inhibits Hh signaling, we analyzed the Hh activity in several models to ICK inhibition. The Sonic hedgehog (Shh)-LIGHT2 NIH 3T3 cells stably express the GLI-driven Firefly luciferase reporter capable of monitoring the Hh pathway activity (32). We inhibited endogenous Ick activity by treatment with FGF2 in Shh-LIGHT2 cells, and analyzed the transactivation of the Hh reporter. At the maximal induction obtained with 100 nM SAG, FGF2 inhibited the luciferase activity by 37% (SI Appendix, Fig. S5A). Similarly, inhibition of Ick activity in NIH 3T3 cells using flavopiridol, AT7519, or lestaurtinib abolished the SAG-mediated GLI1 up-regulation, as did FGF2 (SI Appendix, Fig. S5B). Finally, micromasses derived from murine Ick\(^{-/-}\) limb buds failed to efficiently up-regulate Gli1 and Ptc1 expression and ciliary GLI2 localization upon SAG, similarly to the FGF2-treated control micromasses (SI Appendix, Fig. S5 C and D).
Hh regulation of chondrocyte proliferation in the growth plate cartilage (38). Indeed, poor and mislocalized expression of Hh target genes Gli1, Ptc1, and HoxD13 was found in the growing skeleton of the Ick-/- mice (13, 16). However, the two Ick-/- mouse models had differing ciliary length consequences. Ick-/- mice reported by Chaya et al. (33) had shortened primary cilia, yet the Moon et al. (13) mouse model showed extended primary cilia, demonstrating that both extension and shortening of primary cilia due to ICK insufficiency adversely affects Hh signaling. Similar effects were induced by experimental activation of FGF signaling, when both extension of primary cilia caused by transient FGFR activation, or shortening of cilia induced by constitutive FGF signaling, lead to similar attenuation of cell response to Hh signal (4).

In this study, we demonstrate that FGFR interacts with ICK to regulate cilia length. Several questions remain to be addressed to fully understand the process. First, it is unclear where in cell the FGFR–ICK interaction takes place. Because ICK, 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 agreement with the Moon et al. (13) mouse model showing extended primary cilia, and the cilia length was measured. GFP transfection was used as a control. FGF2 extended primary cilia length in wild-type Ick add-back cells but not in Ick-E80K cells. (H) Human control and Ick-E80K fibroblasts were serum starved and immunostained to visualize cilia. (Scale bar, 1 μm.) Cells were treated with FGF2 and the cilia length was measured. (I) Micromasses produced from limb buds of either Ick-/- (control) or Ick-/- E12 mouse littersates were treated with FGF2 for 24 h and stained with hematxoylin or ARL13B/γ-tubulin. (Scale bars: 1 mm (Left) and 5 μm (Right).) The cilia lengths were measured and graphed. Student’s t test, ***P < 0.01, ****P < 0.001; n.s., nonsignificant.

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. Beacey, Stanford University School of Medicine, Stanford, CA (32). Control and ICK-E80K fibroblasts were established by an International Skeletal Dysplasia Registry, and obtained from the Registry under an approved University of California, Los Angeles human subjects protocol involving informed consent. Cells were propagated in DMEM, supplemented with 10% FBS and antibiotics (Invitrogen). FGF2 was from R&D Systems, SAG from Millipore, leastaurtinib and flavopiridol from Tocris, AT7519 from Selleckchem. Cells were transfected using the FuGENE6 (Promega), Lipofectamine 2000 (Invitrogen), or by electroporation using the

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**Fig. 7.** FGF increases cilia length via ICK. (A) CRISPR/Cas9 targeting of Ick in triploid NIH 3T3 cells. IckCRISPR #1–3, three clones with all three alleles targeted; the positions of deletions are indicated (N.m.d., nonsense mediated decay). (B) Ick expression was analyzed by qPCR, and normalized to Gapdh. (C) Ick protein levels were analyzed by Western blot. (D) 293T cells were transfected with FLAG-tagged wild-type Ick, kinase-dead Ick-E80K or Ick variants harboring in-frame deletions as shown in A, and Western blot for Ick activating phosphorylation (p) at Y159 was used to determine the kinase activity. (E) Cilia in IckCRISPR cells were visualized by ARL13B, acetylated tubulin (AcTu), polyglutamylated tubulin (PolygluTu) or γ-tubulin (γTu) immunostaining. Both long and extremely short cilia signals are shown for IckCRISPR cells. (Scale bar, 1 μm.) Missing AcTu staining is indicated (arrow). (F) IckCRISPR cells were treated with FGF2 for 12 h and the cilia length was measured; black dots, individual cilia; red bars, medians. (G) IckCRISPR #3 cells were transfected with wt Ick or Ick-E80K, treated with FGF2, and the cilia length was measured. GFP transfection was used as a control. FGF2 extended primary cilia length in wild-type Ick add-back cells but not in Ick-E80K cells. (H) Human control and Ick-E80K fibroblasts were serum starved and immunostained to visualize cilia. (Scale bar, 1 μm.) Cells were treated with FGF2 and the cilia length was measured. (I) Micromasses produced from limb buds of either Ick-/- (control) or Ick-/- E12 mouse littersates were treated with FGF2 for 24 h and stained with hematoxylin or ARL13B/γ-tubulin. (Scale bars: 1 mm (Left) and 5 μm (Right).) The cilia lengths were measured and graphed. Student’s t test, ***P < 0.01, ****P < 0.001; n.s., nonsignificant.
cells were sorted using α|β (ab138435; Abcam), GLI3 (sc-33041; Santa Cruz Biotechnology); FLAG (F1804), GST (G1160; Sigma); V5 (R960-27, Santa Cruz) and FLAG (F1804; Sigma) antibodies; FGFR3 (sc-123; Santa Cruz) and FLAG (F1804; Sigma) antibodies; FGFR3 (sc-123), actin (ab8227; Abcam), pFGFR3 (I01-10G; SignalChem), labeled by DyLight 650 (ThermoFisher Scientific). Fluorescence intensities of the peptides were determined with ImageJ.}

**Immunocytochemistry, PLA, and Cilia Length Measurements.** Cells were fixed in 3% paraformaldehyde and incubated with the following antibodies: VS (R960-25), acetylated α-tubulin (232-2700; Invitrogen), FLAG (F1804; Sigma), ARHL3 (17711-1-AP; Proteintech), γ-tubulin (ab11316), pericentrin (ab4448; Abcam), polyglutamylated tubulin (AG-208-0020-C100; Adipogen), SMOC (sc-166685), BS58 (sc-27009), IFTF172 (sc-393833); Santa Cruz), and GLI3 (AF3690; RnD Systems). Duolink PLA (Sigma) was used for PLA, with VS (sc-83849); Santa Cruz) and FLAG (F1804; Sigma) antibodies; GLI3 (sc-123; Santa Cruz) was used to counterstain the transfected cells. AlexaFlour®488/594 secondary antibodies were from Invitrogen. PLA analysis was done in Fiji (fiji.sc/) using maximum projections of z-stacks. Cilia length in 3D was determined as described previously (16).

**MS, Modelung, Animal Experiments, Immunohistochemistry, and Statistics.** Kinase reactions containing FGFR3 and ICK were subjected to reduction, alkylation, and in-solution digestion by trypsin. Samples were analyzed by nanoscale liquid chromatography connected to the tandem mass spectrometer (SRSLc nano 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 Proteome Discoverer software (v.1.4; Thermo) with a Mascot search engine (v.2.4.1; Matrix Science). Quantitative information assessment and phosphopeptide 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
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