Gli2 and Gli3 Localize to Cilia and Require the Intraflagellar Transport Protein Polaris for Processing and Function

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Intraflagellar transport (IFT) proteins are essential for cilia assembly and have recently been associated with a number of developmental processes, such as left–right axis specification and limb and neural tube patterning. Genetic studies indicate that IFT proteins are required for Sonic hedgehog (Shh) signaling downstream of the Smoothened and Patched membrane proteins but upstream of the Glioma (Gli) transcription factors. However, the role that IFT proteins play in transduction of Shh signaling and the importance of cilia in this process remain unknown. Here we provide insights into the mechanism by which defects in an IFT protein, Tg737/Polaris, affect Shh signaling in the murine limb bud. Our data show that loss of Tg737 results in altered Gli3 processing that abrogates Gli3-mediated repression of Gli1 transcriptional activity. In contrast to the conclusions drawn from genetic analysis, the activity of Gli1 and truncated forms of Gli3 (Gli3R) are unaffected in Tg737 mutants at the molecular level, indicating that Tg737/Polaris is differentially involved in specific activities of the Gli proteins. Most important, a negative regulator of Shh signaling, Suppressor of fused, and the three full-length Gli transcription factors localize to the distal tip of cilia in addition to the nucleus. Thus, our data support a model where cilia have a direct role in Gli processing and Shh signal transduction.

Introduction

Cilia are microtubule-based organelles that protrude from the surface of most cells in the mammalian body and are formed through a conserved process termed intraflagellar transport (IFT) [1]. Polaris, the protein encoded by Tg737, is a core component of the mammalian IFT machinery and is required for the formation of all cilia and flagella [2,3]. Mice homozygous for the hypomorphic Tg737

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Abbreviations: E[number], embryonic day [number]; GFP, green fluorescent protein; Gli, Glioma; IFT, intraflagellar transport; MTOC, microtubule organizing center; Ptc, Patched; Ptch, Patched; RT-PCR, reverse transcription PCR; Shh, Sonic hedgehog; ShhN-CM, ShhN conditioned medium; Sufu, Suppressor of fused

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Synopsis

Cilia are small projections extending from the surface of most cells. Research has shown that they are important in diseases such as cystic kidney diseases as well as during the development of many tissues including the limb. More recently, proteins such as Polaris, which is required to build cilia, have been demonstrated to be essential for the regulation of Sonic hedgehog (Shh) signaling, although the mechanism has remained elusive. Precise regulation of Shh signal transduction is important for the proper development of many tissues. Excessive activation of the Shh pathway results in severe developmental defects and has been implicated in certain types of cancer. In the limb, Shh signaling is involved in digit development, and excess signaling leads to the formation of extra digits. The main targets of Shh signaling are the Glioma (Gli) family of transcription factors, and Gli3 has been shown to be processed to a shortened repressor form when Shh signaling is repressed. The localization of the Gli transcription factors and Suppressor of fused, a protein involved in the regulation of Gli protein function, to cilia suggests that the cilia may be an important site for regulation of Shh signal transduction by modulating Gli protein function.

Results

Cilia Are Present on Both Ectoderm and Mesenchyme Cells of the Limb Bud

To determine whether cilia are present on the developing mouse limb bud, we conducted electron microscopic analysis of embryonic day 11.5 (E11.5) limb buds. Using transmission electron microscopy, cilia were found on the mesenchyme. These cilia have a 9 + 0 microtubule structure, which were frequently found in depressions in the cell membrane, and were always closely associated with the Golgi apparatus (Figure 1A–1C). In addition, small vesicular structures were frequently detected near the base of the cilium. Using scanning electron microscopy, we also determined that most, if not all, ectodermal cells exhibit a single cilium (Figure 1D and 1E).

To further confirm the presence of cilia in the limb bud, we conducted immunofluorescence analysis of frozen sections using anti-acetylated α-tubulin, which recognizes stabilized microtubules including the cilium axoneme, and anti-Polaris antisera. The data indicate that Polaris concentrates at the base and distal tip of cilia on both ectodermal and mesenchymal cells as well as in a punctuate pattern overlapping that of acetylated α-tubulin in the axoneme (Figure 1F–1H). In primary cultures of limb bud cells, cilia were found on most cells when visualized with anti-acetylated α-tubulin and anti-Polaris antibodies (Figure 1H). In contrast, the cilia were completely absent from cells isolated from Tg737[^2][^3]-gal mutants (Figure 1I). Domains of stabilized microtubules were still present around the microtubule organizing center (MTOC) from which the cilia would have emerged.

The Hedgehog Signaling Pathway Is Repressed in Tg737[^2][^3]-gal Mutants

In agreement with previous data in the limb and neural tube [18–20], there was no significant expression of two downstream targets of Shh signaling, Ptc1 and Gli1, in Tg737[^2][^3]-gal null mutant limb buds (Figure 2A and 2B). These data suggest that despite normal Shh expression in these mutants, Shh release or reception is impaired because of the loss of Polaris. These results confirm our assessment that the IFT mutant limb phenotype is not due to ectopic activation of the Shh pathway and that the phenotype in
transcription PCR (RT-PCR). While robust response to ShhN-CM was seen in wild-type cells, cells lacking Tg737 showed no increase in the expression of Ptc1 or Gli1 relative to control treated cells (Figure 2C). These data indicate that Polaris is required in Shh responding cells to activate the Shh signaling pathway in the presence of ligand.

Loss of Polaris Results in Altered Gli Activity and Processing

Genetic studies have indicated that IFT function is required for Shh signaling downstream of Ptc1, possibly at the level of Gli function [18–20]. To further explore the connection between Gli activity and Polaris, we used adenoviruses [21] to express the full-length Gli proteins in Tg737 null cells. Previous results have shown that ectopic expression of Gli1 and Gli2 can induce transcription of Shh target genes while Gli3 has been shown to inhibit Gli1-mediated transcription [8,21,22]. As seen in wild-type cells, infection of Tg737Δ2–3β-gal primary limb buds with full-length Gli1 resulted in increased transcription of Ptc1 compared to infection with green fluorescent protein (GFP)—only virus (Figure 3A). This indicates that Polaris function is not required for Gli1-mediated pathway activation. However, infection of Tg737Δ2–3β-gal primary limb bud cells with Gli2-expressing virus failed to induce Ptc1 transcription (Figure 3B) suggesting that Gli2 function requires the activity of Polaris. It is unclear at this time whether the loss of Gli2 function in Tg737Δ2–3β-gal mutants is due to a requirement of Polaris for Gli2 stability or other post-translational regulation. As seen in previous studies, infection of cells with the full-length form of Gli3 was able to repress Gli1-mediated transcription when coexpressed in wild-type cells [22]. However, in cells lacking Polaris, full-length Gli3 failed to repress pathway activation by Gli1, as evidenced by increased Ptc1 expression (Figure 3A).

Gli3 Processing Is Inhibited by Loss of Polaris

The above data raised the possibility that loss of Polaris impaired the conversion of the full-length Gli3 to the

Cells Lacking Polaris Are Unable to Respond to ShhN

To test whether Polaris is required for Shh reception, we isolated cells from Tg737Δ2–3β-gal mutant and wild-type limb buds (E11.5) and cultured the cells in ShhN-CM. The ability of the cells to respond to ShhN was determined by induction of Ptc1 and Gli1 expression using semi-quantitative reverse

Figure 2. Shh Signaling Is Defective in Tg737Δ2–3β-gal Mutants (A and B) In situ hybridization analysis of Ptc1 (A) and Gli1 (B) expression indicates that they are not expressed in the posterior limb buds of Tg737Δ2–3β-gal mutant embryos (E10.5; right panels) as they are in wild-type controls (E10.5, left panels). (C) Incubation of wild-type limb bud cells with ShhN-CM results in upregulation of Gli1 and Ptc1 expression (left lanes) compared to vector conditioned medium, whereas no increase is seen in cells isolated from Tg737Δ2–3β-gal mutant limb buds (right lanes). The relative levels of induction standardized to actin are indicated below each lane. DOI: 10.1371/journal.pgen.0010053.g002

Figure 1. Cilia Are Present on Both Mesenchymal and Ectodermal Cells of the Developing Limb

(A–C) Transmission electron micrographs of limb bud mesenchyme show cilia (arrows) closely associated with the Golgi apparatus (“G”). The cilia exhibited a 9 + 0 structure (C) and are often found in deep depressions in the membranes (B). Frequently, small vesicles are observed fusing or budding with the surrounding membrane (arrowheads in (B) and (C)). (D and E) Scanning electron micrographs of the limb ectoderm show a single cilium (arrows) on nearly all ectodermal cells. (F and G) Immunolocalization of Polaris (red) and acetylated α-tubulin (green) in frozen sections of limb buds shows that Polaris concentrates at the base and tip of the axoneme in both mesenchymal (F) and ectodermal (G) cells. Nuclei are blue. (H and I) In primary cultures of cells isolated from E11.5 limb buds, cilia (arrow in H) are also present when visualized with anti-acetylated α-tubulin (green) and anti-Polaris (red) antisera (H). Cilia are absent on cells isolated from Tg737Δ2–3β-gal mutant limb buds (I); however, the stabilized microtubules were still evident around the basal body region (arrow). The nuclear staining for Polaris is present in the Tg737Δ2–3β-gal cells, indicating that it is nonspecific. Nuclei are blue. DOI: 10.1371/journal.pgen.0010053.g001

Tg737Δ2–3β-gal mutants resembles that of Gli3Δ/−/Shh−/− embryos [13,14].

Cilia are present on both mesenchymal and ectodermal cells of the developing limb (A–C) Transmission electron micrographs of limb bud mesenchyme show cilia (arrows) closely associated with the Golgi apparatus (“G”). The cilia exhibited a 9 + 0 structure (C) and are often found in deep depressions in the membranes (B). Frequently, small vesicles are observed fusing or budding with the surrounding membrane (arrowheads in (B) and (C)). (D and E) Scanning electron micrographs of the limb ectoderm show a single cilium (arrows) on nearly all ectodermal cells. (F and G) Immunolocalization of Polaris (red) and acetylated α-tubulin (green) in frozen sections of limb buds shows that Polaris concentrates at the base and tip of the axoneme in both mesenchymal (F) and ectodermal (G) cells. The nuclei are blue. (H and I) In primary cultures of cells isolated from E11.5 limb buds, cilia (arrow in H) are also present when visualized with anti-acetylated α-tubulin (green) and anti-Polaris (red) antisera (H). Cilia are absent on cells isolated from Tg737Δ2–3β-gal mutant limb buds (I); however, the stabilized microtubules were still evident around the basal body region (arrow). The nuclear staining for Polaris is present in the Tg737Δ2–3β-gal cells, indicating that it is nonspecific. Nuclei are blue. DOI: 10.1371/journal.pgen.0010053.g001

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truncated repressor form. To determine if this was the case, we examined the levels of full-length and processed forms of Gli3 in wild-type and Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) whole embryos (E11.5) by Western blot analysis using Gli3 antiserum (gift of B. Wang). In agreement with previously published results \([19,20]\), there was a marked increase in the ratio of the full-length Gli3 to the processed form of Gli3R in Tg737 mutants (Figure 3C), although some Gli3R is clearly evident. Together, these data suggest that Polaris is required for efficient processing of Gli3.

To determine if the loss of Gli3-mediated repression in Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) mutants was due to defects in processing of full-length Gli3 to the repressor form, we infected primary cells with a truncated form of Gli3 (Gli3R) and analyzed the resulting phenotypes with the genotypes of the embryos. In agreement with previously published results \([19,20]\), there was a marked increase in the ratio of the full-length Gli3 to the processed form, we infected primary cells with a truncated form of Gli3 (Gli3R) and analyzed the processed form of Gli3R in Tg737 mutants (Figure 3C), although some Gli3R is clearly evident. Together, these data suggest that Polaris is required for efficient processing of Gli3.

Partial Loss of Polaris Function Exacerbates the Phenotype of Gli3 Heterozygous Mutants

We addressed the possibility that if Polaris is required for proper Gli3 processing, partial loss of Polaris function as seen with the Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) hypomorphic allele would exacerbate the phenotype of Gli3 heterozygous mice and cause a phenotype that is more reminiscent of Gli3 null mutants. To evaluate this possibility, we crossed Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) heterozygous mice with Gli3\(^{\Delta 2-3^\beta-\text{gal}}\) compound heterozygotes and correlated the resulting phenotypes with the genotypes of the embryos. Heterozygous Gli3\(^{\Delta 2-3^\beta-\text{gal}}\) mice are viable and exhibit a single additional preaxial digit similar to that seen in homozygous Gli3\(^{\Delta 2-3^\beta-\text{gal}}\) mutants [6,12,23]. In contrast, homozygous Gli3\(^{\Delta 2-3^\beta-\text{gal}}\) mutants are nonviable and have 8–10 nonpatterned digits per limb, as is also seen in Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) null mutants [12]. Intriguingly, no viable Gli3\(^{\Delta 2-3^\beta-\text{gal}}\):Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) offspring were obtained (0/64 pups; seven litters). Analysis at earlier developmental stages indicated that the Gli3\(^{\Delta 2-3^\beta-\text{gal}}\):Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) mice die during gestation with severe developmental abnormalities including 6–9 digits per limb, exencephaly, abdominal closure defects, and edema (Figure 4A–4E; data not shown). These phenotypes are not characteristic of Gli3\(^{\Delta 2-3^\beta-\text{gal}}\):Tg737\(^{\Delta 2-3^\beta-\text{gal}}\). To determine if the partial patterning defects in Gli3\(^{\Delta 2-3^\beta-\text{gal}}\):Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) mice were due to a loss of Shh

Figure 3. Gli2 and Full-Length Gli3 Function Is Disrupted in Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) Mutant Cells

(A) Infection of primary limb bud cells (E11.5) with Gli1::GFP expressing adenovirus induces increased Ptch1 transcription in wild-type cells when compared to infection with GFP-only virus (GFP). Coinfection of wild-type cells with Gli1::GFP and Gli3::GFP results in a decrease in the level of Ptch1 expression when compared to cells infected with Gli1::GFP only. As seen in wild-type cells, infection of Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) mutants with Gli1::GFP induced Ptch1 expression. However, full-length Gli3::GFP was unable to suppress Gli1::GFP-mediated induction of Ptch1 in the absence of Polaris (Tg737\(^{\Delta 2-3^\beta-\text{gal}}\)Ptch1). No expression was seen in controls without reverse transcriptase (-RT).

(B) Infection of wild-type cells with a Gli2::GFP expressing adenovirus induced Ptch1 expression; however, in Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) primary limb bud cells, infection with the Gli2::GFP expressing adenovirus failed to induce the pathway, when compared to infection with GFP-only virus (GFP, right lanes). Coinfection of wild-type cells with Gli1::GFP and a truncated Gli3R::GFP indicates that Gli3R is able to repress Gli1-mediated transcription or trafficking to the nucleus.

(C) Western blot analysis of proteins isolated from whole E11.5 wild-type embryos (left lane) shows that Gli3 is predominantly found in the processed repressor form (Gli3R). While some Gli3R is evident in the mutant samples, a large proportion of Gli3 remains unprocessed (Gli3A) in Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) mutant cells (right lane).

(D) Coinfection of wild-type or Tg737\(^{\Delta 2-3^\beta-\text{gal}}\) mutant cells with Gli1::GFP and a truncated Gli3R::GFP indicates that Gli3R is able to repress Gli1-mediated induction of Ptch1.

Numbers below each lane in (A), (B), and (D) indicate the expression level of Ptch1 relative to the actin control for the experiment shown.

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responsiveness, as seen in $T_{g}737^{2–3b-gal}$ mutants, or more closely resembled the phenotype of $Gli3^{Alu}$ mutants, we tested primary cells derived from these embryos for their ability to induce Gli1 expression in response to ShhN-CM. In contrast to $T_{g}737^{2–3b-gal}$ mutants, $Gli3^{Alu}$/$T_{g}737^{phloxph}$ cells responded to ShhN-CM with increased expression of Gli1 when analyzed by quantitative RT-PCR, although at reduced levels compared to wild-type cells (Figure 4F). In agreement with the quantitative RT-PCR data, Gli1 expression was observed in the posterior region of all embryos by in situ hybridization (data not shown). No overt or consistent differences were evident in $Gli3^{Alu}$ or $Gli3^{Alu}$/$T_{g}737^{phloxph}$ embryos when compared to wild-type (data not shown). Together these data indicate that the $Gli3^{Alu}$/$T_{g}737^{phloxph}$ phenotype more closely resembles that of $Gli3$ homozygotes than that of $T_{g}737$ null mutants, which are nonresponsive to ShhN-CM.

Exogenously Expressed Components of the Shh Signaling Pathway Localize to the Cilia

While it is known that IFT is required for Shh signaling [18,19], it remains unclear whether this is due to a requirement for cilia in Shh pathway activation, production of a secondary signal by cilia, or a novel non-ciliary role for IFT. To begin distinguishing between these possibilities, we evaluated the subcellular localization of several key proteins involved in the Shh signaling pathway, including Gli1, Gli2, Gli3, Gli3R, and Sufu relative to cilia and the IFT protein Polaris. In the case of the Gli1, Gli2, Gli3, and Gli3R proteins, localization was determined by infection of primary $T_{g}737^{2–3b-gal}$ mutant and wild-type limb bud cells with adenoviral vectors that express the full-length Gli proteins or the truncated Gli3R fused to GFP [21]. Infections were performed such that greater than 75% of the cells expressed GFP. For these studies, we focused on cells that had low levels of exogenous expression to minimize any effects that over-expression may have on protein localization. For all three full-length Gli proteins, expression was detected in the nucleus in cells that expressed high levels of GFP (Figure 5; data not shown), as reported previously [22]. However, we also detected a small domain of GFP in all cells expressing the tagged protein that was located near the cilium axoneme as visualized with anti-acetylated $\alpha$-tubulin antibodies (Figure 5A–5C). The GFP signals failed to colocalize with $\gamma$-tubulin (basal body marker), indicating that the Gli1::GFP proteins do not localize to the basal body at the base of the cilia (Figure 5E; data not shown). Rather, the GFP signal was found to colocalize with a subdomain of Polaris (Figure 5F and 5G; data not shown). The colocalization of Gli1::GFP with a domain of Polaris, but not with $\gamma$-tubulin, indicates that the full-length Gli proteins concentrate at the tip of the cilium but not at the base. Treatment of infected cells with ShhN-CM did not alter the distribution of Gli1, Gli2, or Gli3 at the distal tips of cilia (data not shown). However, it may be difficult to assess any changes in localization since GFP is fused to the C-terminus of the Gli proteins. Thus, processing that occurs in the case of full-length Gli3 would remove the GFP tag and prevent visualization of the truncated N-terminal form of the protein that traffics to the nucleus.

In contrast to the localization observed for the three full-length Gli proteins, Gli3R::GFP was detected predominantly in the nucleus. We could detect no GFP signal at the distal tip of cilia, suggesting that after processing, Gli3R is released from the cilia or that the cilia targeting domain is located in the C-terminus of Gli3 (Figure 5D). These possibilities are currently being explored.

In $T_{g}737^{2–3b-gal}$ mutant cells that lack cilia, the Gli1::GFP fusion proteins were seen in the nucleus, as observed in wild-type samples. Additionally, Gli3::GFP, Gli2::GFP, and Gli3::GFP were localized around the MTOC, where the cilia would have formed (Figure 5H–5I). In contrast, Gli3R was present mainly in the nucleus and was not detected around the MTOC in either the wild-type or $T_{g}737^{2–3b-gal}$ mutant cells (Figure 5D and 5K). The nuclear localization of the Gli1::GFP proteins in $T_{g}737^{2–3b-gal}$ mutants suggests that Polaris is not required for nuclear import of the Gli transcription factors.

Endogenous Gli3 and Sufu Localization to the Tip of Cilia

To confirm the localization of GFP-tagged Gli3 at the tip of cilia, and to determine if this was the full-length Gli3 protein or only the GFP-tagged C-terminus, we conducted immuno-
fluorescence analysis of endogenous Gli3 in noninfected primary limb cells using Gli3 antisera generated against the N-terminus of the protein (Figure 6). The data indicate that, as seen with exogenously expressed Gli3::GFP, endogenous Gli3 was concentrated at the tip of cilia (Figure 6A; data not shown). Since the Gli3 antiserum recognizes the N-terminus of Gli3, and GFP is fused to the C-terminus in Gli3::GFP virus, the data suggest that it is the full-length form of Gli3 that localizes to the cilium tip.

Since all three Gli proteins localize to the tip of the cilium and to the nucleus, and since Sufu has been shown to directly interact with the Gli proteins [17], we predicted that Sufu would also be present in these two regions of the cell. To explore this possibility, we analyzed the localization of endogenous Sufu by immunofluorescence in primary limb bud cultures. The data confirm that endogenous Sufu colocalized with endogenous Gli3 and with the Gli::GFP fusion proteins, and partially overlapped with Polaris (Figure 6A and 6D; data not shown). It was also present adjacent to acetylated α-tubulin in the cilium axoneme and did not localize with γ-tubulin at the basal body, again indicating that these proteins concentrate at the tip of the cilium (Figure 6B and 6C). A low level of Sufu was also evident in the nucleus but was in a different plane of focus when the Sufu image for Figure 6 was captured. In addition, Sufu was detected in the cytosol; however, whereas the nuclear and cilium tip signals were blocked by preincubation with the immunizing peptide, the cytoplasmic signal remained unchanged (Figure 6E and 6F), suggesting that it may be nonspecific.

Discussion

Cilia are expressed on many different cell types in the mammalian body. They are formed and maintained by a highly conserved process termed IFT, but they perform diverse functions on various cell types [1]. In mammals, cilia have been demonstrated to play a critical role in developmental processes—from left–right axis specification and skeletal patterning to normal kidney, pancreas, and liver physiology—as well as disease processes [2–4,6,24]. Recent data have demonstrated that the IFT proteins, which are necessary for cilia formation, are also required for proper limb and neural tube patterning [6,18–20]. Furthermore, the IFT proteins have been shown to function as part of the Shh signal transduction pathway and in regulating Gli activity [19,20]. Here we show that Gli2 and full-length Gli3 function are disrupted in the Tg737D2–3b-gal cilia mutants. In contrast, our data indicate that Gli1 and a processed form of Gli3 (Gli3R::GFP) (K) are detected only in the nucleus. Mice homozygous for the hypomorphic Tg737apor allele
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Figure 6. Endogenous Sufu and Gli3 Localize to the Distal Tip of the Cilium in Wild-Type Primary Limb Cell Cultures

Sufu (green) and endogenous Gli3 (red) concentrate in the same domain in cultured wild-type limb bud cells (A). As shown for the full-length Gli::GFP proteins, endogenous Sufu does not colocalize with γ-tubulin (red) (B), but is concentrated in a domain at the distal end of the acetylated γ-tubulin staining (red) (C). Sufu also partially overlaps with a domain of Polaris (red) (D) in cultured wild-type limb bud cells. Pre-incubation of anti-Sufu antiserum with the immunizing peptide (E), but not with a nonspecific peptide (F), blocks staining at the distal tip of the cilium (anti-Polaris, red; anti-Sufu, green). Inset in all panels shows Gli3 (A) or Sufu (B–F) staining only in the indicated cilium (arrow).

Materials and Methods

Mouse strains and methods. Tg737Δ2–3b-gal, Tg737m1b and Gli3b/J mice have been previously described [3,4,23]. Tg737Δ2–3b-gal mice were maintained on a mixed FVB × BALBc background and genotyped as described [3]. Gli3b/J mice were maintained on a C57BL/6 background and genotyped as described [3,25]. Analysis of phenotypes for Gli3b/J/Tg737m1b mice was performed on pups or embryos from F1 intercrosses. For staged embryos, noon of the day of the vaginal plug appeared was considered E0.5.

In situ hybridization analysis was performed according to standard protocols [26]. Ptc1 and Gli1 probes were previously described [27,28]. Skeletal stains were performed as described [29].

Cell culture. Shhn-CM and vector-only control conditioned medium were generated as previously described [30]. For induction assays, cells were cultured in a 1:1 mixture of conditioned medium and DMEM + 15% FBS overnight prior to RNA isolation.

Embryos were isolated and identified by phenotype (Tg737Δ2–3b-gal mutants) or by PCR using DNA isolated from yolk sacs. Limb buds for cell culture experiments were removed and treated with 0.25% trypsin in PBS for 15 min at room temperature. Following trypsin treatment, cells were mechanically dissociated and FBS was added to

Table 3. Summary of Gli3 Functions

| Function     | Gli3a/J | Gli3b/J | Gli3Δ3′/Δ3′ | Gli3R | Gli3Δ3′/Δ3′R |
|--------------|---------|---------|-------------|-------|-------------|
| Preaxial polydactyly | ×       | ×       | ×           | ×     | ×           |
| Postaxial polydactyly | ×       | ×       | ×           | ×     | ×           |
| Dorsal defects | ×       | ×       | ×           | ×     | ×           |
| Axial defects | ×       | ×       | ×           | ×     | ×           |

not loss of repressor activity. While processing of Gli2 remains controversial, Gli2 function is disrupted in Tg737Δ2–3b-gal mice, suggesting that IFT or cilia are required for some aspect of Gli2 regulation or activity. This does not appear to involve translocation of the Gli proteins to the nucleus since they are all detectable in the nucleus of Tg737Δ2–3b-gal mutant cells.

In Drosophila, Su(fu) is involved in the negative regulation of Ci (Gli homolog) transcriptional activity by sequestering it in the cytoplasm and targeting it for proteolytic processing to produce a transcriptional repressor [8]. Whether the role of Sufu in targeting Gli proteins for proteolytic processing is conserved in mammals has yet to be determined. Mammalian Sufu has been shown to interact with all three Gli proteins through a conserved SYGH motif in the N-terminus of the Gli proteins in addition to a region in the C-terminus of Gli1, and negatively regulates Gli1 transcriptional activity [16,17]. The colocalization of Sufu and the Gli proteins to the tip of the cilium, along with a requirement for IFT in proper Gli3 processing, suggests that mammalian Sufu may have a similar role in Gli regulation and, furthermore, that proteolytic processing may occur at the tip of the cilium.

Huangfu et al. [18] proposed two possible models for how IFT may regulate Shh signaling, one suggesting the involvement of a cilium-derived signal that is required for Shh pathway activation and a second model in which IFT has two separate functions, one in ciliogenesis and a second one in intracellular transport. While testing these models is hindered by our inability to specifically disrupt cilia formation without also perturbing IFT, the data presented here support a direct role for cilia in Shh pathway regulation. This is based on the localization of multiple components of the Shh pathway in the cilia of wild-type cells, and on altered Gli3 processing and impaired Gli2 function detected in cells lacking this organelle. While we cannot conclusively rule out a non-ciliary function of IFT, we propose that IFT functions to direct and concentrate the Gli proteins, Sufu, and possibly the proteolytic machinery needed for efficient processing of the Gli proteins to a domain located at the distal tips of cilia. In the absence of the cilium, the Gli proteins localize diffusely around the basal body region and fail to undergo normal processing, resulting in their impaired activity.
10%. Cells were collected by centrifugation and plated with DMEM + 15% FBS.

Gli1-GFP, Gli2-GFP, and Gli3-GFP adenovirus constructs encoding C-terminal GFP fusions have been previously described [21]. The Gli3R-GFP adenovirus was generated by replacing the full-length cDNA in the Gli3-GFP vector with the coding region corresponding to amino acids 1–677. This truncated form of Gli3 (Gli3R) has been previously shown to act as a constitutive repressor of Shh signaling [31]. All infections were optimized to produce greater than 75% infection and were done at least three times. To determine if expression levels were consistent between samples, we examined the level of expression of the GliC-GFP genes by RT-PCR using primers specific for the GFP coding region. Similar levels of expression were seen in wild-type and mutant samples under the same infection conditions. All localization data shown are representative of the pattern observed in the majority of the cells from all experiments. An identical pattern of localization was observed in the IMCD mouse kidney cell line. No specific localization of GFP was found for cells infected with GFP control virus only.

RNA isolation and RT-PCR. RNA was isolated using TRIzol (Invitrogen, Carlsbad, California, United States) according to the manufacturer’s instructions. Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. Equal amounts of cDNA were used as templates for PCR with Taq Polymerase (Brinkman Instruments, Westbury, New York, United States) according to the manufacturer’s instructions. Expression levels were calculated by comparing the intensity of the Pch1 or Gli1 PCR product to the actin PCR product in the same reaction using LabWorks 4.0 software (UVP, Upland, California, United States). Primer sequences are available upon request.

Quantitative RT-PCR measurement was performed using the SmartCycler machine (Cepheid, Sunnyvale, California, United States). The Taqman primer and probe sets, for Gli1 and 18S rRNA (TaqMan Assays-on-Demand products), were purchased from Applied Biosystems (Foster City, California, United States). The 18S rRNA gene was used as an internal control. The threshold cycle (Ct) for Gli1 was first normalized to the corresponding 18S rRNA Ct. Relative fold differences were then determined using the $2^{-\Delta\Delta Ct}$ method [32] by comparing the expression levels in ShhN-CM-induced cells to their respective control.

Immunofluorescence. For analysis of cilia in vivo, limb buds were dissected from wild-type embryos (E10.5), embedded in OCT, and snap frozen. Sections of 20 μm were cut and stained as previously described [33] using 0.2% Triton X-100 for permeabilization. Cultured primary cells were fixed, permeabilized, and stained using an identical procedure. Anti-Polarsin polyclonal antibody was generated by Sigma-Geneosys (The Woodlands, Texas, United States) and screened. Western blot analysis and immunofluorescence were performed using Western blot conditions. The antibody recognized a single band of the correct size in basal vector conditioned medium controls. No significant difference in expression levels in ShhN-CM-induced cells to their respective control was observed. The antibody recognized a single band of the correct size in basal vector conditioned medium controls. No significant difference in expression levels in ShhN-CM-induced cells to their respective control was observed.

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Author contributions. CJH and BKY conceived and designed the experiments. CJH, BB, YAS, QZ, and BKY performed the experiments. CJH, YAS, EJM, and BKY analyzed the data. CJH and BKY wrote the paper.

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