Published in final edited form as:
Dev Dyn. 2008 August ; 237(8): 2030–2038. doi:10.1002/dvdy.21551.

Intraflagellar Transport, Cilia and Mammalian Hedgehog Signaling: Analysis in Mouse Embryonic Fibroblasts

Polloneal Jymmiel R. Ocbina[1,2] and **Kathryn V. Anderson**[1]

1 *Developmental Biology Program, Sloan-Kettering Institute, 1275 York Avenue, New York, NY 10065*

2 *Neuroscience Program, Weill Graduate School of Medical Sciences, Cornell University, 445 East 69th Street, New York, NY 10065*

Abstract

Genetic studies in the mouse have shown that Intraflagellar Transport (IFT) is essential for mammalian Hedgehog (Hh) signal transduction. In this study, we take advantage of wild type and IFT mutant mouse embryonic fibroblasts (MEFs) to characterize additional aspects of the relationship between IFT and Hh signaling. Exposure to Sonic hedgehog (Shh) ligand or expression of an activated allele of Smo, SmoA1, activates a Hh reporter in wild-type MEFs, but not in MEFs derived from embryos that lack IFT172 or the Dync2h1 subunit of the retrograde IFT motor. Similarly, decreased activity of either Sufu or PKA, two negative regulators of Hh signal transduction, activates the pathway in wild-type, but not IFT mutant, MEFs. In contrast to wild-type MEFs, Smo is constitutively present in the cilia of *Dync2h1* mutant MEFs. This finding suggests that IFT-dependent trafficking of Hh pathway components through the cilium is essential for their function.

Keywords

Dync2h1; IFT172; Smoothened; Suppressor of fused; Protein kinase A; cytoplasmic dynein

Introduction

Most vertebrate cells have a single primary cilium, a slender projection from the surface of the cell. Cilia and flagella share a common structure, with an axoneme of double microtubules that extend from the basal body, a modified centriole, to form a cellular protrusion (Perkins et al., 1986). Assembly and maintenance of cilia is dynamic: new components are continually incorporated at the tip of the axoneme and broken down to primary units to be degraded. Ciliary components that are synthesized in the cell body are delivered, by a process called intraflagellar transport (IFT), to the distal tip of the axoneme where they are assembled into the axoneme (Johnson and Rosenbaum, 1992). In the absence of IFT, cilia cannot be either built or maintained. IFT particles, which carry cargo to the cilia tip, move along the length of the axoneme using the axonemal microtubules as tracks. Anterograde IFT (from the base of the cilium to the distal tip) is powered by the plus-end-directed heterotrimeric kinesin-II and retrograde IFT (from the tip back to the base) depends on the minus-end-directed dynein motor complex (reviewed in Scholey, 2008).

Recent studies have shown that IFT also has an essential role in the mammalian, but not the *Drosophila*, Hedgehog (Hh) pathway. Hedgehog signaling is essential for many aspects of development in both *Drosophila* and vertebrates (McMahon et al., 2003) and inappropriate

email: E-mail: k-anderson@sloankettering.edu.

activity of the pathway can lead to the development of human tumors (Evangelista et al., 2006). The core of the Hh transduction pathway is conserved in evolution. In both *Drosophila* and mammals, the transmembrane protein Patched (Ptc) is the receptor for Hh ligands, and Patched regulates the activity of a second membrane protein, Smoothened (Smo). Active Smo regulates the action of *Drosophila* Ci and vertebrate Gli transcription factors that control Hh target gene expression. Genetic studies in the mouse demonstrated that several different IFT genes (*Ift172*, *Ift88*, *Kif3a* and *Dync2h1*) are required for activity of the mouse Hh pathway at a step downstream of Smo and upstream of the Gli transcription factors (reviewed in Huangfu and Anderson, 2006).

Experiments in mammalian embryos and cultured cells have demonstrated that components of the Hh signal transduction pathway localize to primary cilia, supporting the hypothesis that cilia are required for Hh signaling. In the absence of Shh, Ptch1 is enriched in primary cilia of cultured NIH-3T3 cells and upon Hh binding, Ptch1 ciliary localization is lost (Rohatgi et al., 2007). In parallel, Smo becomes enriched in primary cilia after Shh treatment, and cilia localization is correlated with Smo function (Corbit et al., 2005). Overexpressed Gli proteins are also enriched in the cilia, both before and after exposure to Shh (Haycraft et al., 2005).

The association of mammalian Hh signaling with cilia highlights important differences between *Drosophila* and mammals in the mechanisms of Hh signal transduction from Smo to Ci/Gli proteins. IFT is not required for *Drosophila* Hh signaling: null mutations in the *Drosophila Kif3a* and *Ift88* homologues, *Klp64D* and *nompB*, disrupt the function of ciliated sensory neurons but not Hh signaling (Ray et al., 1999; Han et al., 2003). Other aspects of the Hh signal transduction pathway also differ between *Drosophila* and vertebrates. In *Drosophila*, signaling from Smo to Ci is regulated by a cytoplasmic complex that includes the serine/threonine kinase Fused (Fu), Suppressor of Fused (Sufu) and the kinesin-like protein Cos2 (Robbins et al., 1997; Sisson et al., 1997). As in *Drosophila*, mammalian Sufu negatively regulates the Hh pathway. However, in contrast to the subtle phenotypes caused by loss of Drosophila *Sufu*, *Sufu* null mouse embryos show a full, ligand-independent activation of the pathway (Cooper et al., 2005; Svard et al., 2006) and knockdown of Sufu in fibroblasts caused strong activation of the pathway (Varjosalo et al., 2006). Endogenous Sufu protein localizes to the distal tips of cilia in cultured fibroblasts (Haycraft et al., 2005), suggesting that it may interact with other pathway components in cilia. In *Drosophila*, Protein Kinase A (PKA) is important for both proteolytic processing of Ci and activation of Smo (Wang et al., 2000b; Jia et al., 2004; Zhang et al., 2004). Vertebrate Gli3 is also a target for PKA phosphorylation (Wang et al., 2000a), but vertebrate Smo lacks the PKA phosphorylation sites present in *Drosophila* Smo (Varjosalo et al., 2006). Mice deficient in PKA activity display a strong activation of the Hh pathway in the neural tube (Huang et al., 2002). This phenotype is stronger than caused by loss of Gli3 (Persson et al., 2002), which suggests that PKA has additional, undefined targets in the mammalian Hh pathway.

Here we use mouse embryonic fibroblast (MEF) cells derived from wild-type and IFT mutant embryos to investigate the relationships between cilia and Hh pathway components. We find that mutant cells that lack cilia (*Ift172* mutants) or that that lack normal activity of the IFT retrograde motor (*Dync2h1* mutants) are unable to activate a Gli-dependent luciferase reporter in response to treatment by Shh or expression of an activated form of Smo. We investigate the relationship between cilia and two negative regulators of the signal transduction pathway, Sufu and PKA, and find that Sufu and PKA depend on the IFT proteins for their activity. Cilia localization is required for Smo function, but experiments in *Dync2h1* mutant cells show that cilia localization of Smo is not sufficient to activate the pathway. Our evidence supports the view that primary cilia provide more than a site for enrichment of Hh pathway components and that dynamic trafficking through the cilium is required for Hh signal transduction.

Results

Normal cilia are required for Hh signaling

Genetic studies have shown that specific IFT proteins are essential for the ability of embryonic tissues to respond to Hh ligands (Huangfu et al., 2003; Huangfu and Anderson, 2005; Liu et al., 2005; May et al., 2005; Haycraft et al., 2007) and that Hh pathway components are enriched in cilia (Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). These findings have led to the hypothesis that cilia are required for Hh signaling. However, additional tests are necessary to demonstrate that Hh signal transduction occurs only within cilia.

Previous studies have suggested that there is a correlation between the presence of cilia and the ability of mammalian cells to respond to Hh ligands. Cilia are present on cultured mammalian cells only when the cells are confluent (Archer and Wheatley, 1971) and mutant mouse embryo fibroblasts (MEFs), in which the pathway is constitutively activated, activate a Hedgehog reporter only when grown at high density (Bailey et al., 2002). We confirmed that the presence of cilia on MEFs derived from e9.5 wild-type embryos correlates with cell density (Fig. 1). We found that 94% (± 5 s.d.) of cells in confluent cultures of MEFs were ciliated, whereas fewer cells in subconfluent cultures were ciliated (<20%, Fig. 1A, left panel). When we transfected wild-type MEFs with a Hh-responsive reporter, which carries 8 tandem copies of a wild-type Gli binding site upstream of a luciferase gene (Sasaki, 1997) and tested the response to exogenous Shh stimulation, we found that efficient induction of the Gli-dependent luciferase reporter by Shh was seen only in cells growing at high densities (Fig. 1A, right panel). Similarly, we found that *Ptch1* cells, like wild-type cells, had cilia and activated the reporter only at confluency (Fig. 1B). Thus in both wild-type MEFs treated with Shh and *Ptch1* mutant MEFs, the Hh pathway is activated only under conditions when cells are ciliated.

Shh-dependent neural cell types are not specified in embryos that lack the activity of IFT172, an IFT complex B protein, or the heavy chain of the retrograde IFT dynein, Dync2h1 (Huangfu et al., 2003; Huangfu and Anderson, 2005; May et al., 2005). We generated MEFs from *Ift172*^{wim} and *Dync2h1*^{ttn} mutant embryos to test directly whether the mutant cells can respond to Shh. The *wim* mutation is an apparently null ENU-induced allele of the IFT complex B component, *Ift172* (Huangfu et al., 2003). *Ift172*^{wim} mutant MEFs do not generate cilia as assayed by acetylated α-tubulin staining or by scanning electron microscopy (Fig. 2C and G). *tian-tian* (*ttn*) is an ENU-induced allele of *Dync2h1* that is caused by an A-to-C substitution in the splice donor site upstream of exon 51; this change should cause an in-frame deletion in the protein that would delete 38 amino acids from the AAA ATPase domain 4 and is therefore likely to strongly disrupt gene activity. We observed that cilia on *Dync2h1*^{ttn} mutant MEFs were 30% shorter than wild-type and had characteristic bulges along the axoneme, similar to the phenotype of cilia in *Dync2h1* mutant embryos (n=18, Fig. 2D and H; Huangfu and Anderson, 2005; May et al., 2005). When assayed at confluency, both *Ift172*^{wim} and *Dync2h1*^{ttn} mutant cells failed to activate the reporter above basal levels, in the same experiment where control wild-type MEFs showed strong activation of the Gli-luciferase reporter (Fig. 2I). These findings indicate that Ift172 and Dync2h1 are required cell autonomously to allow activation of the reporter in response to Shh and support the view that cilia are required for Hedgehog signal transduction.

IFT proteins are required for activity of Smo

The subcellular location of the membrane protein Smo, an essential activator of the Hh pathway, appears to be crucial for its activity. In *Drosophila*, the redistribution of Smo from cytoplasmic stores to the cell membrane controls its activity (Denef et al., 2000; Jia et al., 2004). Similarly, in mammals, Smo becomes enriched in the ciliary membrane after treatment of cultured cells with Shh (Corbit et al., 2005). In the mouse embryo, Smo is enriched in nodal

cilia, where Shh signaling is known to occur (Corbit et al., 2005). Mutant forms of Smo that contain a missense mutation in a putative ciliary localization motif of Smo prevent this ciliary localization and cannot stimulate Hh responses (Corbit et al., 2005). These studies showed that Smo localization to cilia is strongly correlated to its activity.

SmoA1 is an activated allele of *Smo* that causes ligand-independent activation of the Hh pathway (Xie et al., 1998; Taipale et al., 2000). In cultured mammalian MDCK cells, SmoA1 is present in cilia, even in the absence of Hh ligands (Corbit et al., 2005). To test whether this activating Smo mutation can bypass the requirement for cilia, we transfected *SmoA1* into wild type and mutant MEFs and assayed Gli reporter activity. As previously reported, transfection of *SmoA1* into wild-type MEFs caused a robust increase in reporter activity, even in the absence of Shh treatment (Fig. 3A; Taipale et al., 2000). In contrast, expression of *SmoA1* had no detectable effect on Gli reporter activity in either $Ift172^{wim}$ or $Dync2h1^{ttn}$ mutant MEFs. These findings are consistent with the genetic epistasis studies carried out in mutant embryos (Huangfu et al., 2003; Huangfu and Anderson, 2005) and demonstrate that the activated Smo cannot activate the downstream pathway when cilia are absent, as in the $Ift172^{wim}$ mutant cells, or when retrograde IFT is disrupted, as in the $Dync2h1^{ttn}$ mutant MEFs.

Effects of knockdown of Sufu and PKA in IFT mutant cells

Two negative regulators of *Drosophila* Hh signaling, Suppressor of Fused (Sufu) and Protein Kinase A (PKA), act downstream of the membrane protein Smo and upstream of the Ci transcription factors (Preat, 1992; Jiang and Struhl, 1995), the same step in the pathway that, in mammals, depends on cilia (Huangfu et al., 2003). Sufu and PKA are also negative regulators of the mammalian Hh pathway and act downstream of Smo (Huang et al., 2002; Varjosalo et al., 2006; Cooper et al., 2005).

To test whether the function of mouse Sufu depends on cilia, we used shRNAs to knock down Sufu in wild-type and IFT mutant MEFs. As shown previously, knockdown of Sufu in wild-type MEFs caused a three- to four-fold activation of the Gli-dependent luciferase reporter in the absence of Shh (Varjosalo et al., 2006). In contrast, knockdown of Sufu in either $Ift172^{wim}$ or $Dync2h1^{ttn}$ MEFs caused no detectable activation of the reporter (Fig. 3B). Therefore decreased activity of Sufu did not activate the pathway in the absence of cilia. Because Sufu is enriched in cilia (Haycraft et al., 2005), these findings suggest that Sufu may act within cilia to keep the pathway off in the absence of ligand.

Mutations that interfere with cAMP binding to the R1 regulatory subunit of PKA effectively repress kinase activity; these mutations act as dominant negative mutations because they prevent PKA holoenzyme dissociation and block its ability to repress the Hh pathway (Clegg et al., 1987; Epstein et al., 1996). To determine whether decreased PKA activity would activate the Hh pathway in cells without normal cilia, we transfected cells with a dominant negative PKA (dnPKA) construct. We found that the reporter was activated in wild-type MEFs expressing dnPKA (Fig. 3C). In contrast, overexpression of dnPKA in $Ift172^{wim}$ and $Dync2h1^{ttn}$ MEFs did not activate the Hh pathway. These findings suggest that, as with Sufu, the PKA-dependent mechanism of pathway regulation depends on cilia and retrograde IFT.

Smo accumulates in cilia that lack the function of the retrograde IFT motor

To test how mutations that affect cilia structure influence Smo localization, we stained wild type and mutant MEFs with anti-acetylated α-tubulin to visualize cilia and anti-Smo to examine Smo protein distribution. In unstimulated wild-type cells, Smo staining was cytoplasmic (Fig 4A). Consistent with previous reports (Corbit et al., 2005;Rohatgi et al., 2007), we found that Smo was enriched in the cilia of wild-type MEFs treated with Shh (Fig. 4B). In both Shh-

treated and non-treated conditions, *Ift172*^{wim} mutant cells, which lack cilia, showed a cytoplasmic distribution of Smo protein (Fig. 4).

Dync2h1^{ttn} mutant MEFs are ciliated, although the cilia have the bulges characteristic of disrupted retrograde IFT (Fig. 2; Huangfu and Anderson, 2005;May et al., 2005). We found that, even in the absence of Shh treatment, Smo was enriched in the cilia of *Dync2h1*^{ttn} MEFs, and we did not see any further enrichment of Smo in the mutant cilia in response to Shh treatment (Fig. 4). Primary MEFs derived from embryos homozygous for a *Dync2h1* missense allele (*Dync2h1*^{lln}) or a gene trap allele that produces an early truncation (*Dync2h1*^{GT}) also show ciliary enrichment of Smo (Fig. 4 and data not shown; Huangfu and Anderson, 2005). The presence of Smo in the primary cilium of *Dync2h1* mutant MEFs indicates that, even though localization to primary cilia is required for Smo function, the presence of Smo in cilia is not sufficient to activate the pathway. The constitutive presence of Smo in the primary cilium of *Dync2h1* mutant MEFs, which fail to respond to activators of the Hh pathway, argues that retrograde IFT regulates the trafficking of both Smo and of other pathway components that carry out Hh signaling. The data also suggest that, in the absence of Hh ligand, Smo is trafficked through the cilium at a baseline level.

Discussion

In the experiments presented here, we find that four quite different mechanisms that can activate the mammalian Hedgehog pathway all depend on IFT. The Hh pathway reporter is activated by addition of Shh ligand, expression of activated Smoothened, or knockdown of Sufu or PKA activity in wild-type embryonic fibroblasts, but all of these treatments fail to activate the Hh target in fibroblasts that lack either IFT172 or the Dync2h1. It is striking that the phenotypes of *Ift172*^{wim} and *Dync2h1*^{ttn} mutant MEFs are identical in all our assays, even though the *Dync2h1*^{ttn} cells have cilia of nearly normal length, as assayed by both SEM and staining with acetylated α-tubulin. These results support the view that cilia and retrograde IFT trafficking are essential to activate the Hh pathway.

The mechanisms of action of Sufu and PKA in mammalian Hh signaling are not yet clear. Mammalian Sufu interacts directly with Gli proteins, and may tether Gli proteins outside the nucleus, just as *Drosophila* Sufu tethers Ci in the cytoplasm in the absence of ligand (Kogerman et al., 1999; Dunaeva et al., 2003). However, mammalian Sufu has a much stronger phenotype than the *Drosophila* gene and is thought to have roles within the nucleus (Paces-Fessy et al., 2004; Barnfield et al., 2005; Svard et al., 2006). Sufu protein is enriched both in the cilium and in the nucleus (Haycraft et al., 2005), and our data are consistent with the possibility that Sufu tethers Gli proteins in cilia to prevent their release to the nucleus in the absence of ligand. However, our findings do not rule out that Sufu has an additional later function within the nucleus.

Decreased PKA activity strongly activates the mammalian Hh pathway (Huang et al., 2002). PKA acts, at least in part, by priming Gli3 for processing to allow generation of the repressor form of the protein by proteasome-dependent proteolysis (Pan et al., 2006; Wang and Li, 2006). The site where biochemical events that promote Gli3 processing take place is not known. Proteasomes are enriched at the basal body, but none have been found within the cilium (Wigley et al., 1999), suggesting that proteolysis does not take place within the cilium. Our findings indicate that PKA activity in the mammalian Hh pathway depends on cilia, which would suggest that PKA may act within cilia.

Genetic experiments to define the relationships between IFT components, Sufu and PKA would require several generations of mouse breeding, but could be carried out much more quickly in cell culture. Nevertheless, the cell culture experiments have a number of limitations. For

example, previous experiments indicated that simple treatment with shRNAs did not fully inactivate Sufu or fully activate the Hh pathway, presumably due to the stability of the protein (Varjosalo et al., 2006). Therefore our results indicate that a partial loss of Sufu activity that is sufficient to activate the pathway in wild-type cells does not activate the pathway in the absence of cilia or retrograde IFT. Similarly, the MEF experiments allow us to conclude that a decrease in the activity of PKA that is sufficient to activate the pathway in wild-type cells has no effect in IFT mutant MEFs. Analysis of the phenotypes of double mutants with null alleles of Sufu, PKA and IFT mutants will provide definitive information on pathway relationships in vivo. In addition, there are clear cell-type specific differences in the requirement for some cilia proteins in the embryo. For example, Shh signaling is disrupted to different extents along the rostrocaudal axis of *Dync2h1* mutant embryos (Huangfu and Anderson, 2005). Such cell-type specific differences in the relationship between retrograde IFT and Shh signaling can only be studied *in vivo*.

We find that Smo accumulates in the cilia of the *Dync2h1* mutant cells in the absence of Shh. This suggests that Smo continually traffics through cilia in the absence of Hh ligand, and accumulates to high levels within the cilium when retrograde IFT is disrupted. Our findings contrast with the report of May et al., who did not detect either Smo or acetylated α-tubulin in the cilia of the embryonic node in *Dync2h1* mutant embryos (May et al., 2005). Because we find that Smo accumulates in the cilia of all three *Dync2h1* mutants examined, we suggest that the difference between the experiments may reflect the greater ease of detection of ciliary Smo in cultured cells than in embryonic tissues.

Our experiments in MEFs suggest that Shh may act by modulating the kinetics of Smo transit through the cilium, rather than by regulating an on/off switch of Smo localization. Shh may increase the rate of delivery of Smo-containing vesicles to the base of the cilium, where Smo is transferred to an anterograde IFT-dependent trafficking mechanism. Once at the tip, Smo may be released, like other IFT cargo, and there it can interact with other Hh pathway components localized at the cilia tip. In either the presence or absence of Shh ligand, if retrograde transport is blocked, both Smo and modified Gli proteins fail to move out of the cilium. The consequence of this is that Smo and Gli proteins both remain at the cilia tip and the nuclear pathway is not activated. More complex models are also possible: for example, Shh could act by decreasing the rate of retrograde IFT, thereby increasing the amount of time Smo can associate with other pathway components at the cilia tip and promoting downstream signaling events. Direct measurements of the rates of Smo trafficking and of anterograde and retrograde IFT in the presence or absence of Shh would, in principle, distinguish between these hypotheses.

Experimental Procedures

Primary Mouse Embryonic Fibroblasts

MEFs were isolated from e9.5 wild type, *Ptch1*, *wim/IFT172*, and *ttn/Dync2h1* mutant mouse embryos and grown on tissue culture plates. MEFs were kept in growth medium of High Glucose DMEM, 0.05 mg/ml Penicillin, 0.05 mg/ml Streptomycin, 2mM L-Glutamine, and 10% Fetal Bovine Serum. At confluence, cells were split 1:3 and kept for up to 7 passages. To induce cell cycle arrest and allow the growth of cilia, growth medium was replaced with 0.5% Fetal Bovine Serum for 48 hrs.

Immunohistochemistry

Cells were grown to confluency on gelatin-treated coverslips or on 8-well chambered slides (Lab-Tek II). For Shh induction, cells were cultured in Shh-conditioned media (diluted 1:4) as described previously (Taipale et al., 2000). After 48 h, cells were fixed with 4%

Paraformaldehyde (PFA) in PBS for 15 minutes and washed 3 times with PBS. Fixed cells were then placed in blocking solution (PBS with 1% v/v Normal Goat Serum and 0.1% v/v Triton X-100) for 30 minutes. To stain for cilia, we used a mouse antibody against acetylated α-tubulin (1:1000, Sigma), which is an established marker to identify cilia in fixed samples. The goat antibody against Smo (1:200, Santa Cruz) was used to detect Smo protein in cultured cells. Primary antibodies were diluted in blocking solution and used to stain cells at 4°C ON. After washing 3 times in PBS, Alexa-coupled secondary antibodies were added in blocking solution at a dilution of 1:500 for 1 h at room temperature. DAPI was added in the final washes and used as a counterstain for nuclei. Samples were mounted in Vectashield and coverslipped. Confocal images were acquired using an LSM510 microscope (Carl Zeiss MicroImaging, Thornwood, NY). Confocal datasets were analyzed using the Volocity software package (Improvision).

Scanning Electron Microscopy

For scanning electron microscopy (SEM), cells were grown on gelatin-treated 12mm round coverslips and serum-starved as described above. To fix cells for SEM, cells in low-serum media were washed with DMEM without serum 3 times and gradually replaced with 2% Glutaraldehyde in DMEM without serum and fixed in this solution for 30 min at room temperature. Cover slips were transferred to a fresh Petri dish containing 2% Glutaraldehyde in 0.1M Na Cacodylate pH 7.2 with 0.1M sucrose and fixed for a further 1.5 h at room temperature. Samples were dehydrated in an ethanol series and processed for critical point drying and coating. Samples were processed and observed according to standard procedures on a Zeiss Supra 25 Field Emission Scanning Electron Microscope.

Constructs

A EcoRI/AvrII fragment from a full length Smo cDNA (IMAGE: 6850065) was subcloned into a pcDNA3.1(+) backbone using the EcoRI and XbaI sites. To create the SmoA1 mutation (Xie et al., 1998), a Trp539Leu mutation was introduced to the wild type Smo construct using the Quikchange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). A cDNA of the RIα regulatory subunit of the PKA holoenzyme was obtained from the IMAGE database (2648357) and a EagI/ScaI fragment was subcloned into a pcDNA3.1(-) backbone using the NotI/EcoRV sites. We induced three mutations (Gly200Glu, Gly324Asp and Arg332His; Clegg et al., 1987) using the Quickchange method (Stratagene, La Jolla, CA, USA). The constructs for the Sufu shRNAs as well as the scramble shRNA were a generous gift from the Taipale lab and were described previously (Varjosalo et al., 2006).

Luciferase Reporter System Assays for Hedgehog Signaling

Wild-type, *Ptch1*, *IFT172*^{wim}, and *Dync2h1*^{ttn} MEFs were seeded in 24-well tissue culture dishes at an initial density of 1.0×10^5 cells/cm^2. After 18−24 h, we transfected the cells with a total of 250 ng DNA per well using the Fugene 6 (Roche) transfection reagent (3:1 ratio of reagent to DNA). To measure Shh response, we transfected MEFs with 8× 3'Gli-BS-luciferase reporter (40%, w/w DNA), Renilla luciferase (pRL-TK, Clontech; 10%), and the plasmid of interest or balanced with pcDNA3.1 (50%). After reaching confluency, the growth medium was changed to low-serum medium (0.5% FBS) and cultured for 48 h. When applicable, low-serum medium was supplemented with Shh conditioned medium (conditioned media used at a 1:4 dilution) that had been aspirated from ecR-Shh Hek293 cells (ATCC, CRL-2782; ref. Taipale et al., 2000) stimulated with 1μm Muristerone A (Invitrogen). Cells were lysed and processed for firefly luciferase reading using the Dual-Luciferase Reporter Assay System (Promega) on a LMaxII 384 (Molecular Devices). All reporter assays were normalized for transfection efficiency using Renilla luciferase values. All assays were done in quadruplicate in at least three independent experiments.

Acknowledgments

We thank Nina Lampen for assistance with SEM, and the Sloan-Kettering molecular cytology core facility for expert technical assistance during confocal imaging. We thank Jussi Taipale (National Public Health Institute, Helsinki, Finland) for the Sufu shRNA constructs, Luca Cartegni (Memorial Sloan Kettering Cancer Center, New York) for use of their luminometer, Danwei Huangfu for helpful discussions on the experiments, and Sarah Goetz, Karel Liem, Nina Matova and Scott Weatherbee for helpful comments on the manuscript. Genome sequence analysis used Ensembl and associated databases.

Grant sponsor: NIH

Grant number: NS044385.

References

Archer FL, Wheatley DN. Cilia in cell-cultured fibroblasts. II. Incidence in mitotic and post-mitotic BHK 21-C13 fibroblasts. J Anat 1971;109:277–292. [PubMed: 5105129]

Bailey EC, Milenkovic L, Scott MP, Collawn JF, Johnson RL. Several PATCHED1 missense mutations display activity in patched1-deficient fibroblasts. J. Biol. Chem 2002;277:33632–33640. [PubMed: 12072433]

Barnfield PC, Zhang X, Thanabalasingham V, Yoshida M, Hui CC. Negative regulation of Gli1 and Gli2 activator function by Suppressor of fused through multiple mechanisms. Differentiation 2005;73:397–405. [PubMed: 16316410]

Clegg CH, Correll LA, Cadd GG, McKnight GS. Inhibition of intracellular cAMP-dependent protein kinase using mutant genes of the regulatory type I subunit. J Biol Chem 1987;262:13111–13119. [PubMed: 2820963]

Cooper AF, Yu KP, Brueckner M, Brailey LL, Johnson L, McGrath JM, Bale AE. Cardiac and CNS defects in a mouse with targeted disruption of suppressor of fused. Development 2005;132:4407–4417. [PubMed: 16155214]

Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF. Vertebrate Smoothened functions at the primary cilium. Nature 2005;437:1018–1021. [PubMed: 16136078]

Denef N, Neubuser D, Perez L, Cohen SM. Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell 2000;102:521–531. [PubMed: 10966113]

Dunaeva M, Michelson P, Kogerman P, Toftgård R. Characterization of the Physical Interaction of Gli Proteins with SUFU Proteins. Journal of Biological Chemistry 2003;278:5116–5122. [PubMed: 12426310]

Epstein DJ, Marti E, Scott MP, McMahon AP. Antagonizing cAMP-dependent protein kinase A in the dorsal CNS activates a conserved Sonic hedgehog signaling pathway. Development 1996;122:2885–2894. [PubMed: 8787761]

Evangelista M, Tian H, de Sauvage FJ. The hedgehog signaling pathway in cancer. Clin Cancer Res 2006;12:5924–5928. [PubMed: 17062662]

Han YG, Kwok BH, Kernan MJ. Intraflagellar transport is required in Drosophila to differentiate sensory cilia but not sperm. Curr Biol 2003;13:1679–1686. [PubMed: 14521833]

Haycraft CJ, Banizs B, Aydin-Son Y, Zhang Q, Michaud EJ, Yoder BK. Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. PLoS Genet 2005;1:e53. [PubMed: 16254602]

Haycraft CJ, Zhang Q, Song B, Jackson WS, Detloff PJ, Serra R, Yoder BK. Intraflagellar transport is essential for endochondral bone formation. Development 2007;134:307–316. [PubMed: 17166921]

Huang Y, Roelink H, McKnight GS. Protein kinase A deficiency causes axially localized neural tube defects in mice. J Biol Chem 2002;277:19889–19896. [PubMed: 11886853]

Huangfu D, Anderson KV. From the Cover: Cilia and Hedgehog responsiveness in the mouse. Proc Natl Acad Sci U S A 2005;102:11325–11330. [PubMed: 16061793]

Huangfu D, Anderson KV. Signaling from Smo to Ci/Gli: conservation and divergence of Hedgehog pathways from Drosophila to vertebrates. Development 2006;133:3–14. [PubMed: 16339192]

Huangfu D, Liu A, Rakeman AS, Murcia NS, Niswander L, Anderson KV. Hedgehog signalling in the mouse requires intraflagellar transport proteins. Nature 2003;426:83–87. [PubMed: 14603322]

Jia J, Tong C, Wang B, Luo L, Jiang J. Hedgehog signalling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase I. Nature 2004;432:1045–1050. [PubMed: 15616566]

Jiang J, Struhl G. Protein kinase A and hedgehog signaling in Drosophila limb development. Cell 1995;80:563–572. [PubMed: 7867064]

Johnson KA, Rosenbaum JL. Polarity of flagellar assembly in Chlamydomonas. J Cell Biol 1992;119:1605–1611. [PubMed: 1281816]

Kogerman P, Grimm T, Kogerman L, Krause D, Undén AB, Sandstedt B, Toftgård R, Zaphiropoulos PG. Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nat Cell Biol 1999;1:312–319. [PubMed: 10559945]

Liu A, Wang B, Niswander LA. Mouse intraflagellar transport proteins regulate both the activator and repressor functions of Gli transcription factors. Development 2005;132:3103–3111. [PubMed: 15930098]

May SR, Ashique AM, Karlen M, Wang B, Shen Y, Zarbalis K, Reiter J, Ericson J, Peterson AS. Loss of the retrograde motor for IFT disrupts localization of Smo to cilia and prevents the expression of both activator and repressor functions of Gli. Dev. Biol 2005;287:378–389. [PubMed: 16229832]

McMahon AP, Ingham PW, Tabin CJ. Developmental roles and clinical significance of hedgehog signaling. Curr Top Dev Biol 2003;53:1–114. [PubMed: 12509125]

Paces-Fessy M, Boucher D, Petit E, Paute-Briand S, Blanchet-Tournier MF. The negative regulator of Gli, Suppressor of fused (Sufu), interacts with SAP18, Galectin3 and other nuclear proteins. Biochem J 2004;378:353–362. [PubMed: 14611647]

Pan Y, Bai CB, Joyner AL, Wang B. Sonic hedgehog signaling regulates Gli2 transcriptional activity by suppressing its processing and degradation. Mol Cell Biol 2006;26:3365–3377. [PubMed: 16611981]

Perkins LA, Hedgecock EM, Thomson JN, Culotti JG. Mutant sensory cilia in the nematode Caenorhabditis elegans. Dev Biol 1986;117:456–487. [PubMed: 2428682]

Persson M, Stamataki D, te Welscher P, Andersson E, Böse J, Rüther U, Ericson J, Briscoe J. Dorsal-ventral patterning of the spinal cord requires Gli3 transcriptional repressor activity. Genes Dev 2002;16:2865–2878. [PubMed: 12435629]

Preat T. Characterization of Suppressor of fused, a complete suppressor of the fused segment polarity gene of Drosophila melanogaster. Genetics 1992;132:725–736. [PubMed: 1468628]

Ray K, Perez SE, Yang Z, Xu J, Ritchings BW, Steller H, Goldstein LS. Kinesin-II is required for axonal transport of choline acetyltransferase in Drosophila. J Cell Biol 1999;147:507–518. [PubMed: 10545496]

Robbins DJ, Nybakken KE, Kobayashi R, Sisson JC, Bishop JM, Therond PP. Hedgehog elicits signal transduction by means of a large complex containing the kinesin-related protein costal2. Cell 1997;90:225–234. [PubMed: 9244297]

Rohatgi R, Milenkovic L, Scott MP. Patched1 regulates hedgehog signaling at the primary cilium. Science 2007;317:372–376. [PubMed: 17641202]

Sasaki H. A binding site for Gli proteins is essential for HNF-3beta floor plate enhancer activity in transgenics and can respond to Shh in vitro. Development 1997;124:1313–1322. [PubMed: 9118802]

Scholey JM. Intraflagellar transport motors in cilia: moving along the cell's antenna. J Cell Biol 2008;180:23–29. [PubMed: 18180368]

Sisson JC, Ho KS, Suyama K, Scott MP. Costal2, a novel kinesin-related protein in the Hedgehog signaling pathway. Cell 1997;90:235–245. [PubMed: 9244298]

Svard J, Henricson KH, Persson-Lek M, Rozell B, Lauth M, Bergstrom A, Ericson J, Toftgard R, Teglund S. Genetic elimination of suppressor of fused reveals an essential repressor function in the Mammalian hedgehog signaling pathway. Dev Biol 2006;10:187–197.

Taipale J, Chen JK, Cooper MK, Wang B, Mann RK, Milenkovic L, Scott MP, Beachy PA. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. Nature 2000;406:1005–1009. [PubMed: 10984056]

Varjosalo M, Li SP, Taipale J. Divergence of Hedgehog Signal Transduction Mechanism between Drosophila and Mammals. Dev Biol 2006;10:177–186.

Wang B, Fallon JF, Beachy PA. Hedgehog-regulated processing of Gli3 produces an anterior/posterior repressor gradient in the developing vertebrate limb. Cell 2000a;100:423–434. [PubMed: 10693759]

Wang B, Li Y. Evidence for the direct involvement of {beta}TrCP in Gli3 protein processing. Proc Natl Acad Sci U S A 2006;103:33–38. [PubMed: 16371461]

Wang G, Amanai K, Wang B, Jiang J. Interactions with Costal2 and suppressor of fused regulate nuclear translocation and activity of cubitus interruptus. Genes Dev 2000b;14:2893–2905. [PubMed: 11090136]

Wigley WC, Fabunmi RP, Lee MG, Marino CR, Muallem S, DeMartino GN, Thomas PJ. Dynamic association of proteasomal machinery with the centrosome. J. Cell Biol 1999;145:481–490. [PubMed: 10225950]

Xie J, Murone M, Luoh SM, Ryan A, Gu Q, Zhang C, Bonifas JM, Lam CW, Hynes M, Goddard A, Rosenthal A, Epstein EH, de Sauvage FJ. Activating Smoothened mutations in sporadic basal-cell carcinoma. Nature 1998;391:90–92. [PubMed: 9422511]

Zhang C, Williams EH, Guo Y, Lum L, Beachy PA. Extensive phosphorylation of Smoothened in Hedgehog pathway activation. Proc Natl Acad Sci USA 2004;101:17900–17907. [PubMed: 15598741]

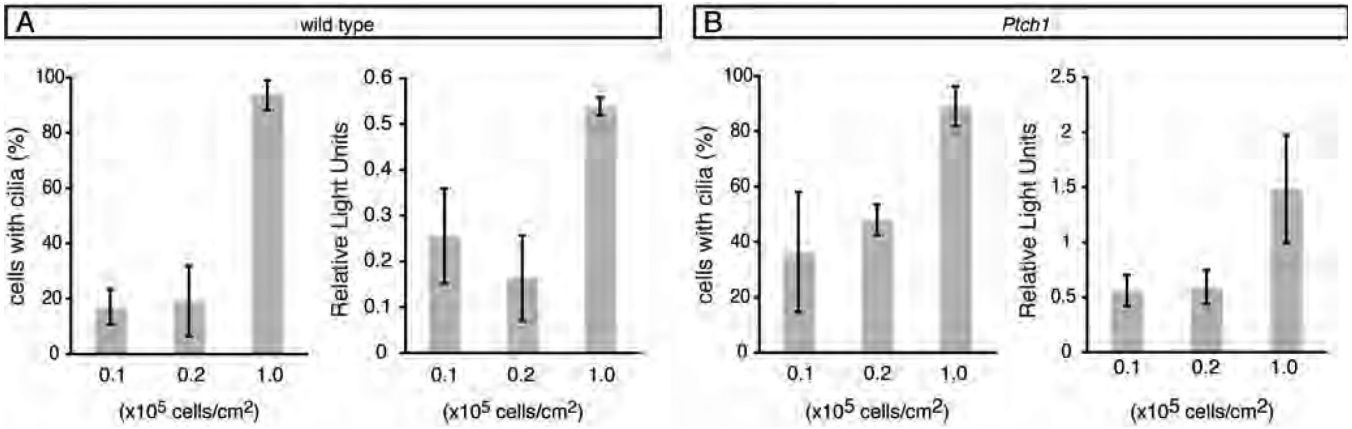

Figure 1. The presence of cilia correlates with Hedgehog responsiveness in wild-type (A) and *Ptch1* (B) mouse embryonic fibroblasts (MEFs)

A. (Left panel) Most wild-type MEFs grown at low density lacked cilia, whereas confluent MEFs almost always grew primary cilia. Wild-type MEFs were initially plated at 1.0, 0.2 and 0.1×10^5 cells/cm^2 in 24-well plates. (Right panel) Hedgehog responsiveness was assayed by transfecting wild type MEFs with Renilla luciferase transfection control and a Hh-responsive Gli-luciferase reporter, then stimulating with Shh-conditioned media (Materials and Methods). The activation of the reporter in response to Shh treatment correlates with cell density, and therefore with the presence of primary cilia on the cells. **B**. *Ptch1* MEFs grow cilia and activate the reporter in a similar cell-density dependent manner in the absence of Shh treatment. Data are mean ± s.d.

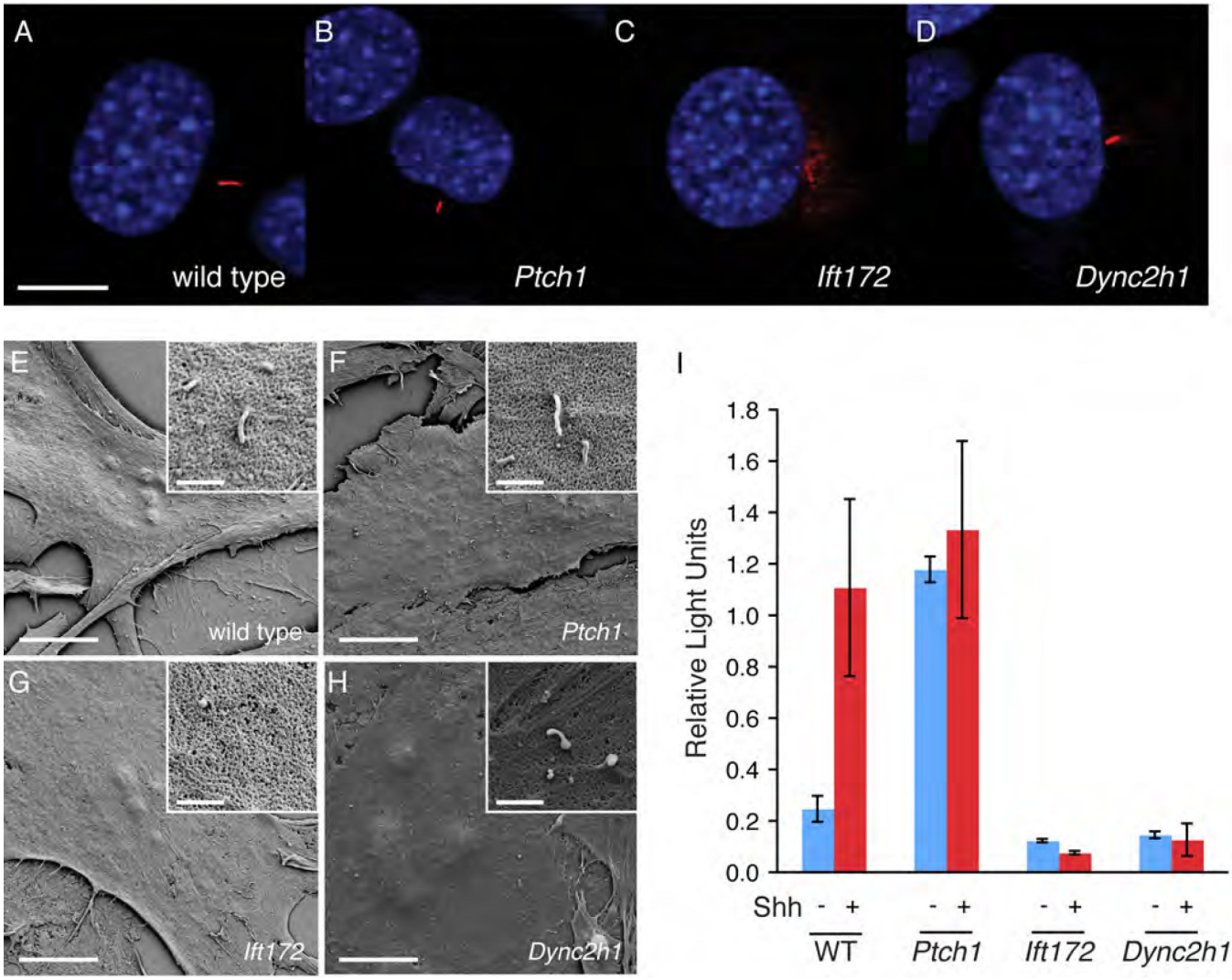

Figure 2. Characterization of cilia phenotypes in *Ift172*^{wim} and *Dync2h1*^{ttn} mutant MEFs

A-D. Immunofluorescent staining of wild type and mutant MEFs for acetylated α-tubulin (red) and DAPI (blue) show that although *Ift172*^{wim} cells do not possess cilia (**C**), mutants that lack the heavy chain of the retrograde IFT motor, Dync2h1, are able to grow cilia (**D**). Scale bar = 5μm. **E-H**. HIgher magnification views of MEFs using scanning electron microscopy. *Ift172*^{wim} MEFs lack cilia (**G**). *Dync2h1*^{ttn} MEFs are able to grow primary cilia (**H**). but their morphology is abnormal compared to WT (**E**) and *Ptch1* (**F**) primary cilia. From 18 *Dync2h1* mutant cilia examined, the mutant cilia were shorter (565 ± 20nm) than wild type (800 ± 30nm). Each mutant cilia had a bulb-like structure along the length of the axoneme. Scale bars, 5μm and 2μm (inset). **I**. Activation of the Gli-luciferase reporter in wild type and mutant MEFs. Wild type cells show robust activation of the reporter in response to Shh treatment. As expected, *Ptch1* mutant MEFs mutant activate the reporter even in the absence of ligand. In contrast, *Ift172*^{wim} and *Dync2h1*^{ttn} MEFs show no activation of the reporter in response to Shh treatment. Data are mean ± s.d.

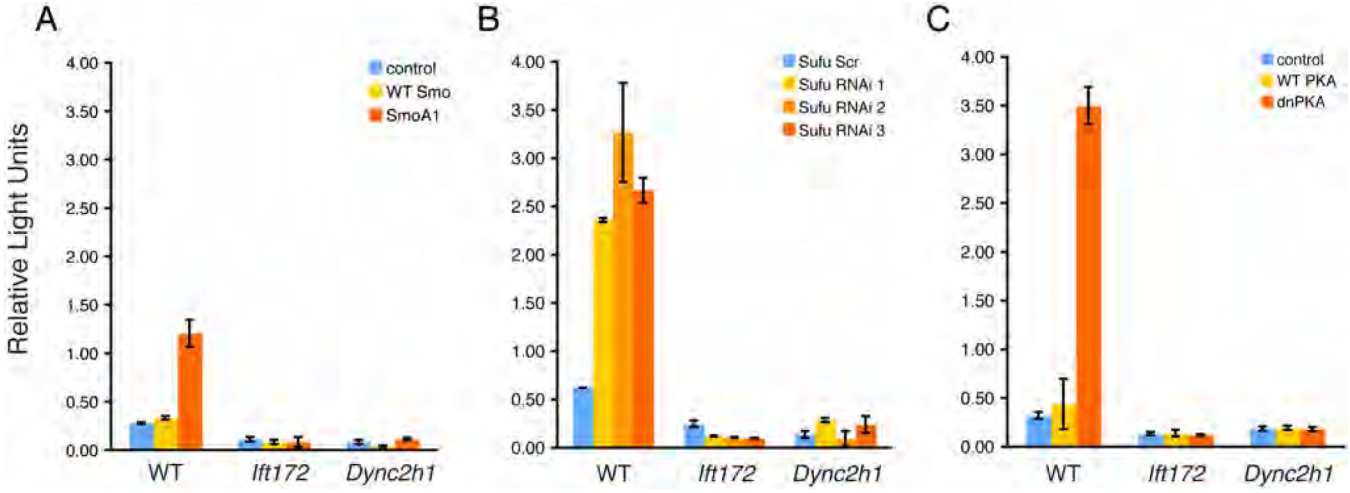

Figure 3. Smo, Sufu and PKA depend on IFT proteins for activity

A. Expression of an activated form of Smo, SmoA1, activates the Gli-luciferase reporter in wild-type, but not *Ift172*^{wim} or *Dync2h1*^{ttn} mutant cells. **B**. Expression of a dnPKA construct activates the Hh pathway in wild type MEFs, whereas *Ift172*^{wim} and *Dync2h1*^{ttn} MEFs are unresponsive to this treatment. **C**. Expression of shRNAs against Sufu in wild type MEFs causes strong activation of the Gli-luciferase promoter. Knockdown of Sufu in MEFs that have a mutation in *Ift172* or *Dync2h1* had no effect on reporter activity. Data are mean ± s.d.

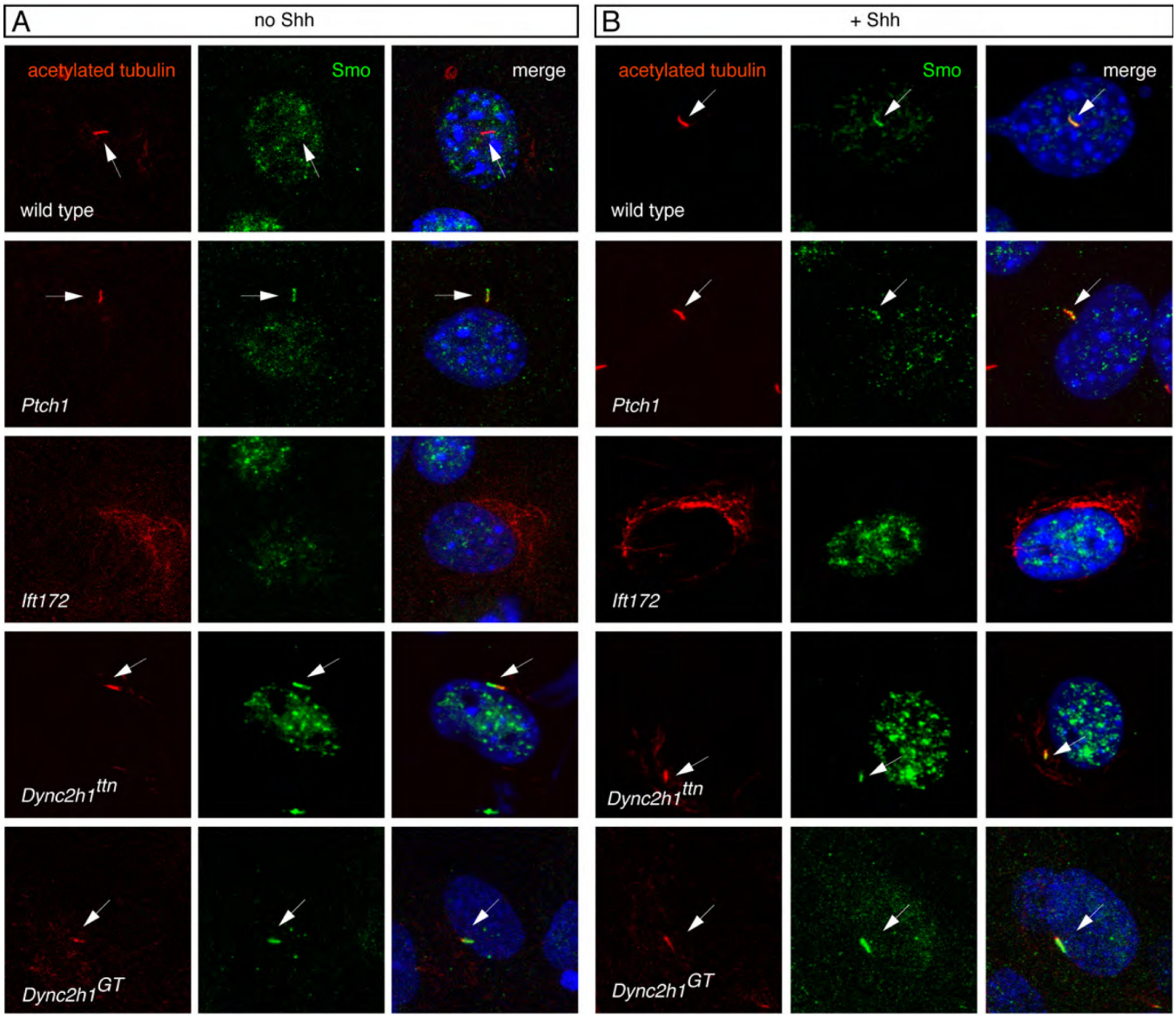

Figure 4. Smoothened localization to cilia is not sufficient to activate the Hh pathway A-B

Localization of Smo (green) in cilia of wild type, *Ptch1*, *Ift172*^{wim}, *Dync2h1*^{ttn} and *Dync2h1*^{GT} MEFs in the absence (**A**) or presence (**B**) of Shh ligand. Acetylated α-tubulin (red) was used to identify cilia and DAPI (blue) was used to label nuclei. In the absence of Shh (**A**), Smo is found in the cytoplasm of wild-type MEFs and is localized to the cilium following 48 hours treatment with Shh (**B**). In MEFs that lack the Hh receptor *Ptch1*, Smo is enriched in cilia regardless of Shh presence. *Ift172*^{wim} MEFs, which do not grow cilia, show cytoplasmic staining of Smo in the presence or absence of Shh. Constitutive enrichment of Smo in the primary cilium was observed in *Dync2h1*^{ttn} and *Dync2h1*^{GT} MEFs, independent of Shh treatment.