Opening new doors: Hedgehog signaling and the pancreatic cancer stroma

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Hedgehog-stimulated chemotaxis is mediated by Smoothened located outside the primary cilium

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ABSTRACT

Regulation of the Hedgehog (Hh) pathway relies on an interaction of two receptors. In the absence of Hh, Patched1 (Ptch1) inhibits the pathway. Binding of the ligand Hh to Ptch1 stimulates the localization of the activating receptor Smoothened (Smo) to the primary cilium, which is required for the transcriptional Hh response. Hh can also induce chemotaxis through a nontranscriptional pathway. We assessed the effects of defective ciliary localization of Smo on its subcellular trafficking and ability to mediate chemotactic signaling. Cells expressing mutants of Smo that could not localize to the primary cilium or cells lacking the primary cilium showed altered intracellular trafficking of Smo, and, in response to Hh or Smo agonists, decreased transcriptional signaling and enhanced chemotactic responsiveness. Thus, the ciliary localization machinery appears to transport Smo to subcellular sites where it can mediate transcriptional signaling and away from locations where it can mediate chemotactic signaling. The subcellular localization of Smo is thus a crucial determinant of its signaling characteristics and implies the existence of a pool of Smo dedicated to chemotaxis.
**INTRODUCTION**

The Hedgehog (Hh) pathway is involved in many inductive events in the developing embryo, the maintenance of tissue integrity in adult organisms, and tumorigenesis [1]. In amniotes, there are three related ligands that can activate this pathway, Sonic (Shh), Desert (Dhh), and Indian (Ihh) Hedgehog. The current working model for Hh pathway activation in vertebrates is that the binding of Shh to the receptor Patched1 (Ptch1) results in an increased localization of Smoothened (Smo) to the primary cilium, a cellular appendage shaped by the microtubule cytoskeleton [2-5]. Additional proteins involved in mediating the response downstream of Smo are also localized to the primary cilium, and the trafficking of Smo to and from the cilium correlates with the transcriptional response mediated by Hh-dependent transcription factors of the Gli family. Thus, in this model of Hh signaling the primary cilium is critical for the transcriptional response stimulated by Shh. Loss of the machinery involved in transport to and from the primary cilium is associated with an attenuation or loss of the Gli-mediated response to Shh, supporting the model [2, 4, 6].

Furthermore, several mouse mutants deficient for cytoskeleton-associated proteins necessary for primary cilium formation show a range of Shh-related phenotypes [7-9], generally resulting from a loss of ligand-mediated modulation of pathway activity through the Gli transcription factors [9]. For example, cells from mice deficient in the kinesin II motor protein Kif3A lack well-organized primary cilia, and Kif3A−/− embryos display a phenotype consistent with an incomplete transcriptional response to Shh [8, 10, 11]. It is hypothesized that, in mice lacking primary cilia, Smo cannot function normally to activate the Gli transcription factors.

The intracellular domain of Smo contains a ciliary localization domain (CLD). The activation of the Gli transcriptional response to Smo either by Shh or by small molecule agonists, such as SAG, results in and also relies on the localization dynamics of Smo to and from the primary cilium [5, 12, 13]. Several Smo mutants, such as the SmoΔCLD mutant, which contains two mutated amino acids in the CLD (W549A, R550A), and the SmoC151Y mutant, which has a point mutation in the N-terminal extracellular cysteine-rich domain [2, 14], fail to localize to the primary cilium in response to Shh, and these mutants cannot mediate the Gli-mediated Shh response.

Besides activating a transcriptional response, Shh can also act as a cellular chemoattractant [15, 16] and can mediate pathfinding of commissural axons and retinal ganglion axons [17-21]. The chemotactic Shh response does not require de novo transcription or translation, nor does it require the function of Gli proteins [16, 22-24]. The chemotactic Shh response, however, does require Smo [18, 25]. The requirement for Smo in both the transcriptional and chemotactic responses suggests that a bifurcation of the Shh response into a transcriptional and a chemotactic branch occurs at, or downstream of Smo, but upstream of Gli activation. Because chemotaxis requires rapid and localized reorganization of the cytoskeleton, it seems unlikely that Shh-induced localization of Smo to the primary cilium is necessary for Shh-mediated chemotactic signaling.
Binding of Shh to Ptch1 causes a redistribution of Smo to different intracellular locations not all of which include the primary cilium [26, 27], but if these different distributions of Smo mediate distinct signaling remains unexplored. Here, we report that defective ciliary localization motifs in Smo or defective ciliogenesis resulted in altered intracellular trafficking of Smo to sites other than the primary cilium. These altered itineraries correlated with reduced capacity for Shh-mediated transcriptional signaling, but enhanced chemotactic responsiveness. This suggests that the ciliary localization machinery plays a role in the transport of Smo to sites where it can mediate transcriptional signaling, and away from sites where it can mediate chemotactic signaling. Therefore, we propose a model in which Smo localization is a crucial determinant of the transcriptional or chemotactic response to Shh.

RESULTS

Kif3A affects Smo trafficking outside the primary cilium

We analyzed Smo dynamics and trafficking by expressing various mutant forms of Smo or wild-type Smo (SmoWT) in Smo−/− immortalized mouse embryo fibroblasts (MEFs) or in Kif3A−/− MEFs, which lack a well-organized primary cilium due to the loss of the kinesin II motor protein Kif3A. These cell-based systems allow us to assess the effect of defective ciliary localization on Smo localization and function. In Smo−/− MEFs wild-type Smo (SmoWT) localized to the primary cilium (Fig. S1A), consistent with earlier observations [13]. Both SmoΔCLD and SmoC151Y mutants showed impaired localization to the primary cilium (Fig. S1B, C). An oncogenic form of human SMO (SMOM2), which stimulates a transcriptional Shh response independent of ligand [28, 29], localized to the primary cilium of Smo−/− MEFs (Fig. S1D). Western blotting indicated that all of the mutants were expressed at similar quantities after transfection (Fig. S1E). These mutant forms of Smo enable the investigation of the effects of defective ciliary localization on subcellular localization of Smo and Shh responsiveness.

Although Smo traffics in and out of the primary cilium, the molecular machinery responsible for controlling this process remains unresolved [12], and these events probably initiate outside the primary cilium. As a consequence, the critical interactions between Smo and the trafficking machinery responsible for transport into the cilium likely occur outside the cilium as well. To investigate how the presence or absence of the machinery responsible for the formation and maintenance of primary cilia affected Smo trafficking, we transfected wild-type (Kif3A+/+) or Kif3A−/− MEFs with SmoWT or SmoΔCLD, and we assessed the distribution of Smo in cells treated with cycloheximide and chloroquine (Fig. 1A, B). Chloroquine prevents acidification of endosomes, thus preventing early to late endosome maturation, and this compound has been used previously to study interaction dynamics of Ptc1 and Smo, and trafficking of these proteins into late endosomes [26, 30]. Blocking translation with cycloheximide prevents newly synthesized protein from obscuring the fraction of proteins that is recycling to the cell surface following endocytosis. Thus, the change in Smo localization in response to chloroquine represents the fraction of this protein that was internalized from the plasma membrane. We quantified the distribution
Smo localization dictates Hh signaling outcome

of Smo by classifying the different intracellular localization patterns observed (Fig. 1A, B). In the *Kif3A*<sup>+/−</sup> cells, SmoWT was predominantly localized in perinuclear vesicular structures following 3 hours of chloroquine treatment (Fig. 1A, blue arrow ‘2’); whereas, in a fraction of cells SmoΔCLD was detected in large vesicles (Fig. 1A, red arrow ‘3’). In the absence of *Kif3A*, the distribution of SmoWT was similar to that of SmoΔCLD (Fig. 1B). The distribution of SmoΔCLD was the same, in large vesicles, following 3 hours of chloroquine treatment in both the *Kif3A*<sup>+/−</sup> (Fig. 1A) and *Kif3A*<sup>−/−</sup> (Fig. 1B) cells. To determine if the differences in the localizations of SmoWT and SmoΔCLD reflected a difference in the rate at which these two proteins trafficked, we quantified the distributions of the two proteins over a 24-hour time course of chloroquine treatment (Fig. 1C, D). SmoWT showed localization to large vesicles in only a small percentage of cells measured (Fig. 1C), whereas this vesicular localization phenotype of SmoΔCLD under these conditions was almost complete (Fig. 1D). This suggests that both the primary cilium and the CLD on Smo regulate the events that underlie trafficking of Smo from the plasma membrane to endosomes.

To more accurately define the different intracellular localizations observed for the different forms of Smo, we stained for markers of distinct intracellular sorting routes. Following treatment with chloroquine, we observed co-labeling of Rab11 and SmoWT (Fig. 1E), suggesting that SmoWT trafficked through a Rab11-positive compartment, which is involved in trafficking towards the basal body of the primary cilium [31] [32]. Although a subpopulation of SmoWT was localized to LAMP1-positive vesicles, which are late endosomes and lysosomes, (Fig. 1F), quantification of the colocalization in cells displaying a class 2 phenotype indicated that this population was smaller than the population in the Rab11-positive structures. The percentage of colocalizing pixels from at least two cells over 2 experiments was 12.7% and 20.6% for Rab11, and 2.5% and 3.0% for LAMP1. We observed little co-labeling of SmoΔCLD and Rab11 (Fig. 1E) in cells displaying a class 3 phenotype (2.4% and 1.1%), but did detect more of this mutant in LAMP1-positive vesicles 11.5% and 9.3%), indicating that in these cells SmoΔCLD preferentially traffics to late endosomes and lysosomes (Fig. 1F). Because Rab11 has been implicated in trafficking towards the basal body of the primary cilium [31], we examined the effect of stable knockdown of Rab11 in Smo<sup>−/−</sup> MEFs on Smo trafficking. Knockdown of Rab11 combined with expression of SmoWT diminished, but did not eliminate, the ciliary localization of SmoWT in the presence or the absence of the agonist SAG (Fig. S2). Because ciliary localization was only reduced and not abolished, either other proteins can mediate trafficking of Smo to the cilium or the knockdown was insufficient to completely block trafficking.

The chloroquine-induced accumulation of SmoΔCLD in endosomes suggests that its itinerary included the plasma membrane. Therefore, we tested by cell surface biotinylation if Smo localization to the plasma membrane was affected by lack of Kif3A or the absence of the CLD. For these experiments, the cells were not treated with chloroquine or cycloheximide. We observed two distinct forms of Smo that were labeled by surface biotinylation, indicating appearance at the plasma membrane, with apparent molecular mass of 110-kD and 95-kD (Fig. 1G). Although both forms were detected in cells expressing SmoWT and in the *Kif3A*<sup>−/−</sup>
Figure 1. Reduced ciliary localization machinery correlates with altered intracellular itineraries of Smoothened. (A-B) *Ki3A*Δ+/+ and *Ki3A*Δ− MEFs were transfected with SmoWT or SmoΔCLD and treated with 1 µg/ml cycloheximide and 100 µM chloroquine (CQ) for 3 hours. Smo was visualized by immunofluorescence, and its localization divided in three classes; (1) unperturbed localization in intracellular compartments and membrane, indicated by grey arrow in images and grey fill in pie graphs; (2) perinuclear accumulation indicated in blue; (3) widespread vesicular localization indicated in red. The fraction of cells with each Smo localization was...
Smo localization dictates Hh signaling outcome

Quantified and the mean of >60 cells in five independent experiments is shown in the pie graphs. Scale bar, 20 μm. (C–D) Quantification of the fraction of cells showing each of the three localizations in Kif3A+/- MEFs transfected SmoWT or SmoΔCLD and treated with 1 μg/ml cycloheximide and 100 μM chloroquine for the times indicated. Data represent the mean of >60 cells in two independent experiments. (E–F) Analysis of the colocalization of SmoWT or SmoΔCLD transfected into Kif3A MEFs treated with 1 μg/ml cycloheximide and 100 μM chloroquine for 3 hours with Rab11 or LAMP1. (G) MEFs were transfected with SmoWT or SmoΔCLD and GFP. Surface biotinylation was performed, and isolated proteins were analyzed by Western blot. Shown are Western blots of surface-labeled Smo, and Smo and GFP in aliquots of the total lysates. (H) Samples were prepared as in (G), except the lysates were treated with Endo H to assess glycosylation status. Shown are Western blots for surface-labeled Smo. The dotted line indicates parts of the membrane that are not shown. The experiments shown in panels G and H are representative of three experiments.

In contrast, SmoΔCLD was predominantly detected as the 95-kD form and the relative amount of the 95-kD form increased in Kif3A-/- cells expressing SmoWT (Fig. 1G). Endoglycosidase H (Endo H) is an enzyme that cleaves oligosaccharides on proteins that have not yet been fully processed within the endoplasmic reticulum (ER) and Golgi system, and thus allows us to distinguish between proteins that have passed through this intracellular route and those that have not; therefore, we treated the lysates with Endo H to explore if Smo present at the cell surface had been fully processed through the ER and Golgi. The 95-kD form of SmoWT and SmoΔCLD was sensitive to Endo H treatment, which decreased the apparent molecular mass to 84-kD, the predicted size of the protein backbone (Fig. 1H). However, the more extensively glycosylated 110-kD form of SmoWT was largely Endo H resistant. Endo H-sensitive forms of Smo were suggested to be ER-localized [33], but the biotinylation assays showed that this form was detectable at the cell surface and was more abundant for SmoΔCLD and SmoWT in the absence of Kif3A. Loss of either Kif3A (and thus the primary cilium) or the CLD resulted in the presence of partially glycosylated Smo at the cell surface, indicative of an altered intracellular itinerary of Smo. The altered localization and intracellular itineraries of Smo may explain why in Kif3A+/- embryos the transcriptional response to Shh is affected and why SmoΔCLD fails to mediate a transcriptional response to Shh.

For the SmoC151Y mutant, we observed a different phenotype from that of SmoΔCLD. Chloroquine triggered a redistribution of SmoC151Y in both Kif3A+/+ and Kif3A-/- MEFs (Fig. 2A, B). Similar to SmoWT, SmoC151Y colocalized with Rab11 (28.9% and 59.4% colocalizing pixels quantified in 6 cells over two experiments), but not LAMP1 (Fig. 2C, D; 5.6% and 3.9% colocalizing pixels). Surface biotinylation experiments with Kif3A+/+ and Kif3A-/- MEFs revealed that SmoC151Y was present at the membrane as the partially glycosylated form, similar to that of SmoΔCLD, (Fig. 2E), and this form was Endo H sensitive (Fig. 2F). The total amount of SmoC151Y in Kif3A-/- MEFs relative to that in wild-type MEFs (Fig. 2E) appeared greater, which may indicate that, in Kif3A-/- MEFs, a smaller fraction of SmoC151Y trafficked to the cell surface. These data and the biotinylation studies with SmoWT and SmoΔCLD indicated that Kif3A facilitated trafficking of partially glycosylated forms of Smo to the plasma membrane.
Smo mutants unable to localize to the primary cilium preferentially mediate Hedgehog chemotaxis

To determine if the differences in intracellular trafficking of Smo and the Smo mutants had functional consequences, we measured the transcriptional response to the agonist purmorphamine [34] mediated by the different forms of Smo in cells expressing a Gli-luciferase reporter [35]. SmoWT restored the transcriptional response of Smo^−/− MEFS; whereas SmoΔCLD and SmoC151Y failed to restore a transcriptional response to purmorphamine (Fig. 3A), which is consistent with a previous report [14]. In contrast, both SMOM1 and SMOM2 produced transcriptional responses that were independent of agonist, with SMOM2 producing the largest response (Fig. 3A). These results with the various mutants are consistent with the previously established correlation between ciliary localization of Smo and its ability to activate the transcriptional response [2].
To test the requirement for ciliary localization of Smo in the non-transcriptional Shh response, we assessed the ability of cells expressing SmoWT, SmoΔCLD, or SmoC151Y to migrate towards sources of ligand (Shh or purmorphamine) with a modified Boyden chamber [16]. Dose-response experiments have shown that the concentration that produces the half maximal migration (EC_{50}) is ~200 nM purmorphamine and ~20 nM SAG [33]. These values are similar to those observed for the transcriptional response (500 nM purmorphamine, 3 nM SAG) [33, 36]. Expression of SmoWT (Fig. 3B-E), SmoΔCLD, or SmoC151Y restored the ability of Smo-/- MEFs to migrate towards Shh or purmorphamine (Fig. 3C, D). Migration to fetal calf serum (FCS) served as a control for nonspecific cell motility or motility defects. Expression of SMOM2 resulted in impaired migration to FCS, but both SMOM1 and SMOM2 exhibited a chemotactic response to purmorphamine or recombinant ShhN (Fig. 3C, D). Inclusion of purmorphamine in both chambers, thus eliminating the gradient, did not result in net cell movement of cells transfected with any form of Smo (Fig. 3C). We noted that the Smo mutants that were unable to localize to the primary cilium mediated chemotaxis to purmorphamine more effectively than did SmoWT (Fig. 3C) and we confirmed the enhanced chemotactic responsiveness of cells expressing SmoΔCLD relative to those expressing SmoWT in response to recombinant ShhN (Fig. 3D) or SAG (Fig. 3E). These results suggested that localization to the primary cilium was not required for Shh-stimulated chemotaxis, and that the altered intracellular localization of these mutants may enhance the chemotactic response to Shh.

Cells expressing SMOM1 or SMOM2 cells showed a chemotactic response similar to that mediated by SmoWT, demonstrating that these mutants can be activated by purmorphamine to mediate chemotaxis. This is in apparent contradiction with the idea that these alleles are constitutively active [28, 29]. However, it may be possible that these mutants are constitutively active for mediating the transcriptional response, but retain ligand-regulated chemotactic signaling.

To test if activating mutations in Smo are dominant over a defective CLD, we introduced the mouse equivalent of the M2 mutation, A1 [28], into SmoΔCLD (SmoCLD-A1) (Fig. S3A). Expression of SmoCLD-A1 failed to rescue the defective ciliary localization (Fig. S3B) or the transcriptional activity (Fig. S3C). However, this mutation did not diminish the enhanced chemotactic signaling capacity conferred by the mutated CLD (Fig. S3D).

We investigated the functional conservation of Smo signaling responses (transcriptional pathway activity and chemotactic pathway activity) by expressing Smo from other species in the Smo-/- MEFs and monitoring the outputs of these two functional pathways. We transfected Smo-/- MEFs with Smo from zebrafish (Danio rerio) or fruit fly (Drosophila melanogaster). Smo from fish or fly failed to mediate activation of the transcriptional pathway (Fig. 3F); however, both proteins activated the chemotactic pathway, and Smo from the fruit fly was more effective than mouse Smo (Fig. 3G). This exchangeability implies that chemotactic signaling by Smo is either more robust (less affected by small mutations or intracellular organization defects) than the transcriptional response or perhaps more evolutionarily conserved than transcriptional signaling.
To assess if previously described signaling mediators between Smo and the cytoskeleton were responsible for the observed chemotaxis, we performed inhibitor experiments on Smo-/- MEFs transfected to express SmoWT. The alkaloid cyclopamine binds to and inhibits Smo [37]. Pretreatment with this inhibitor diminished migration to purmorphamine, confirming a requirement for Smo (Fig. 3H). Signaling downstream of Smo to the cytoskeleton has previously been described to rely on Smo-mediated activation of G proteins and is inhibited by pertussis toxin [38]. Indeed, pertussis toxin inhibited migration to purmorphamine (Fig. 3H). The leukotriene synthesis machinery is also involved in Shh-stimulated chemotaxis [25]. We found that the leukotriene receptor antagonist inhibitor MK-571 inhibited chemotaxis to purmorphamine (Fig. 3H), suggesting that an autocrine leukotriene signaling loop contributed to Shh-stimulated chemotaxis.

Shh-mediated axon guidance relies on the activation of Src family kinases [21] and this response is blocked by the kinase inhibitor PP2. Neither PP2 nor the related inhibitor PP1 reduced the migration of SmoWT-expressing MEFs to purmorphamine (Fig. 3H). We detected phosphorylation of Src in cells expressing SmoΔCLD, but not in those expressing SmoWT (Fig. S4). Together, these results suggested that Smo-mediated chemotactic signaling in MEFs does not involve Src, but that SmoΔCLD may activate signaling events involving Src.

**Cells without primary cilia retain their chemotactic response to Shh**

The enhanced chemotactic responsiveness found in cells transfected with Smo mutants that fail to localize to the primary cilium indicated that the absence of the primary cilium should not compromise Smo-mediated chemotactic signaling. We assessed the consequences for transcriptional and chemotactic Shh signaling of genetic perturbations resulting in defective formation of the primary cilium. Staining Kif3A-/- MEFs for acetylated α-tubulin confirmed that these cells did not have an obvious primary cilium (Fig. 4A). Although many cells had distinct puncta of acetylated α-tubulin, we never observed the rod-like appearance typical for primary cilia. Kif3A-/- MEFs were significantly impaired in their transcriptional response to purmorphamine (Fig. 4B); however, these cells displayed a chemotactic response to purmorphamine, which exceeded that of wild-type MEFs (Fig. 4C). The chemotactic response to purmorphamine was not affected by actinomycin D and thus did not require transcription, but was inhibited by cyclopamine and therefore depended on Smo. Kif3A-/- MEFs also exhibited an increased chemotactic response to recombinant ShhN (Fig. 4D) and SAG (Fig. 4E). These results are consistent with the requirement of the primary cilium for the transcriptional response, and also demonstrated that Smo signaling in the absence of the primary cilium favored Shh chemotaxis apparently.

Tg737orpk is a hypomorphic allele of the gene encoding the intraflagellar transport protein Ift88. Cells homozygous for this allele have severely shortened primary cilia [39], and in mouse models, this results in impaired Shh-mediated patterning of the developing neural tube [8, 40]. Consistent with these observations, we found that immortalized Tg737orpk MEFs had shortened cilia compared to the cilia of wild-type MEFs (Fig. 4F), and showed a reduced transcriptional response to purmorphamine (Fig. 4G). Similar to the Kif3A-/- MEFs, Tg737orpk MEFs were...
Figure 3. Defective ciliary localization enhances chemotactic signaling by Smo. (A) Activation of the transcriptional response pathway in MEFs transfected with indicated forms of Smo and Gli-luciferase reporter. Luciferase activity was measured and corrected for cotransfected Renilla luciferase following 2 µM purmorphamine (purm.) or DMSO (control) stimulation for 16 hours. Shown is average fold change relative to the control-treated vector-transfected cells ± SEM, n=6. Statistical differences from SmoWT are indicated. (B) MEFs were transfected with SmoWT or vector only (pcDNA3.1) and transferred to a modified Boyden chamber. Chemotaxis was stimulated by the addition of 2 µM purmorphamine or 2 nM ShhN to the lower compartment. The RFU of cells exposed to medium containing no attractant (control for basal migration) was subtracted from the RFU of cells exposed to chemoattractant. RFU, relative fluorescence unit, corresponds to about five cells; data were collected every 2 min for 99 cycles. (C) Migration of Smo−/− MEFs transfected with vector or indicated mutant forms of Smo. Data shown are the average ± SEM, n≥3. Statistical differences from SmoWT are indicated. FCS has fetal calf serum as the attractant; purm. no gradient has 2 µM concentration of purmorphamine in both chambers; purm. has 2 µM in the lower chamber only. (D) Migration of Smo−/− MEFs transfected with vector or indicated mutant forms of Smo toward 2 mM recombinant ShhN. Data shown are the average ± SEM of ≥3 experiments, statistical differences from SmoWT are indicated. (E) Migration of Smo−/− MEFs transfected with vector or indicated mutant forms of Smo toward 500 nM SAG. Data shown are the average ± SEM of ≥3 experiments, statistical differences from SmoWT are indicated. (F) Activation of the transcriptional response pathway in Smo−/− MEFs transfected with mouse (M. musculus), zebrafish (D. rerio), or fruitfly (D. melanogaster) Smo using the Gli-luciferase reporter. Data shown are the average ± SEM, n=3. Statistical differences from M. musculus Smo are indicated. (G) Migration toward 2 µM purmorphamine of Smo−/− MEFs transfected with Smo from the indicated species. Statistical differences from M. musculus Smo are indicated. (H) Smo−/− MEFs were transfected with SmoWT, pretreated with the indicated inhibitors for 10 min, and chemotaxis to purmorphamine was assessed. Ctrl represents no pretreatment. Cyclopamine, 5 µM; pertussis toxin (PTX), 1 µM; MK-571, 5 µM, PP1 and PP2, 10 µM. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t test.
significantly more efficient in chemotaxis in response to purmorphamine, recombinant Shh, or SAG than their wild-type counterparts (Fig. 4H-J). The enhanced chemotactic response observed for these mutant MEFs was not due to a general increase in migratory capacity, because migration towards FCS was unaffected by the loss of the primary cilium (Fig. 4C, H).

**Figure 4.** Cells with defective ciliary function show enhanced chemotactic signaling by Smo. (A) The presence or absence of primary cilia on Kif3A+/+ and Kif3A−/− MEFs was visualized by immunofluorescence for acetylated α-tubulin. Scale bar, 20 µm. (B) Activation of the transcriptional response pathway in Kif3A MEFs transfected with Gli-luciferase reporter and stimulated 2 µM purmorphamine or DMSO (control). Shown is average fold change relative to the control-treated Kif3A+/+ cells ± SEM, n=3. Statistical difference to the purmorphamine-stimulated Kif3A+/+ MEFs is indicated. (C) Chemotaxis to purmorphamine or FCS of Kif3A MEFs was assessed and shown as average fluorescence ± SEM, n=4. Where indicated cells were exposed to 500 ng/mL actinomycin D (act. D) or 10 µM cyclopamine (cyA) for 10 min. prior to placement in the Boyden chamber. Statistical difference to purmorphamine-stimulated chemotaxis of Kif3A+/+ MEFs is indicated by the red asterisks. Statistical difference to purmorphamine-stimulated chemotaxis of Kif3A−/− MEFs is indicated in the absence of inhibitors is indicated by the gray asterisks. (D) Chemotaxis of Kif3A MEFs to recombinant ShhN. Data shown are the average fluorescence ± SEM, n=4. Statistical difference to the rShhN-stimulated Kif3A+/+ MEFs is indicated. (E) Chemotaxis of Kif3A MEFs to SAG. Data shown are the average fluorescence ± SEM, n=4. Statistical difference to the SAG-stimulated Kif3A+/+ MEFs is indicated. (F) Primary cilia on wild-type or Tg737 orpk MEFs were visualized by immunofluorescence for acetylated α-tubulin. Scale bar as in panel A. (G) Activation of the transcriptional response pathway in Tg737 MEFs transfected with Gli-luciferase reporter, stimulated with purmorphamine, and analyzed as described for panel B. Data are the mean ± SEM, n=6. Statistical difference to the purmorphamine-stimulated wild-type MEFs is indicated. (H) Chemotaxis of Tg737 orpk or wild-type MEFs to purmorphamine in the presence or absence of the inhibitors as described in panel C. Statistical difference to purmorphamine-stimulated chemotaxis of Tg373 wild-type MEFs is indicated by the red asterisks. Statistical difference to purmorphamine-stimulated chemotaxis of Tg373 orpk MEFs in the absence of inhibitors is indicated by the gray asterisks. (I-J) Chemotaxis Tg737 orpk or wild-type MEFs to recombinant ShhN or SAG. Data are the mean ± SEM, n=3. Statistical differences between wild-type and Tg373 orpk are indicated. *P < 0.05, **P < 0.01, ***P < 0.001 by Student’s t test.
Smo localization dictates Hh signaling outcome

Ciliary localization-impaired Smo enhances neurite outgrowth

Axonal pathfinding and cell migration are related events that share molecular mechanisms and guidance molecules [41]. Neurite extension of embryonic stem (ES) cells stimulated to undergo neural differentiation serves as an in vitro assay for assessing neural guidance signaling. In neuralized, ES cell-derived embryoid bodies (EBs), exposure to Shh results in the upregulation of Isl1 and Isl2 (collectively referred to as Isl1/2), which are established markers of motor neuron differentiation [42]. We evaluated the projection of neurites by staining for class III β-tubulin (Tuj1) in neutralized EBs exposed to Shh. In neuralized EBs, the induction of motor neurons is a consequence of activating the transcriptional Shh response, whereas the Shh-induced projection of neurites from neurons is independent of Gli activity and transcription [25]. The distance between crossing neuritis is a relative measure of neurite network density and this distance negatively correlates with network density, enabling quantification of neurite outgrowth in very dense reticula where tracing individual neurite projections is impossible [25].

Together, retinoic acid (RA) and SAG induce ventral neutralization of EBs [42, 43]. However, EBs derived from Smo-/- ES cells did not show a robust ventral neutralization in response to RA and SAG as measured by neurite length (Fig. 5A) or the number of Isl1/2-positive cells (Fig. 5B). We were unable to confer additional responsiveness to these cells by transfecting Smo and for further experiments, EBs were derived from Smo +/- ES cells transfected with vector, SmoWT, or SmoΔCLD. Transfected Smo constructs were expressed at similar amounts as determined by densitometry on Western blot (abundance of SmoΔCLD was 102% of SmoWT, Fig. S5). SAG stimulated an increase in the length and number of neurite projections (Fig. 5C) and in the proportion of Isl1/2-positive cells (motor neurons) (Fig. 5D). Both the projection of neurites (Fig. 5E) and the proportion of Isl1/2-positive cells (Fig. 5F) produced in response to SAG was enhanced by SmoWT. Consistent with the enhanced ability of SmoΔCLD to mediate chemotactic Shh signaling in fibroblasts, we found that ES cells expressing SmoΔCLD showed a strong induction of neurite outgrowth in response to SAG (Fig. 5G), but these cells did not show an increase in the number of Isl1/2-positive motor neurons compared to vector-transfected EBs (Fig. 5H). This indicated that SmoΔCLD mediated SAG-induced neurite outgrowth, but not the SAG-induced transcriptional response.

Neurite extension and motor neuron differentiation are both Shh-dependent, but can be uncoupled pharmacologically with leukotriene inhibitors, which selectively block the Shh-dependent neurite extension response [25]. We used this method to confirm that the neurite outgrowth in transfected Smo +/- EBs was separate from differentiation and that they represented independent Shh responses. Treatment of Smo +/- EBs transfected with SmoΔCLD with MK-886, a 5-lipoxygenase inhibitor, specifically disturbed neurite extension without affecting differentiation (Fig. 5I). This underscores that Isl1/2 and Tuj1 stainings specifically represent the transcriptional or the chemotactic Shh response in these EBs. Together, the data showed that defective ciliary localization of Smo enhanced neurite outgrowth and suggest that Smo localized outside the primary cilium can mediate this process.
Figure 5. Smo localized outside the primary cilium enhances neurite outgrowth. (A) Smo−/− ES cells were transfected with vector. After 24 hours, the cells were plated to form embryoid bodies (EBs) and after 3 days the EBs were replated without inducers, or neuralized and ventralized by addition of 1 µM retinoic acid (RA) and 200 nM SAG. After an additional 4 days in culture, class III β-tubulin-positive neurites were visualized and the distance between crossing neurites was measured and plotted as the relative frequency. Frequency data represent >10 measurements for at least three EBs in two experiments. Scale bar, 100 µm. (B) Induction of motor neurons

- Smo+/− + SmoWT
- no inducers
- RA/SAG

Average: 22.1
Average: 14.4

Average: 23.4
N.S.
Average: 10.8***
Avg: 23.7 N.S.
Avg: 7.9***

- Smo−/− + Smo−/−
- no inducers
- RA/SAG

Average: 35.3
Average: 23.3

Average: 23.4***
Average: 10.8***

Average: 23.7***
Avg: 7.9***

- Isl1/2+ cells/EB

- Class III β-tubulin

- Distance between crossing neurites (µm)

- Isl1/2+ cells/EB

- Class III β-tubulin

- Distance between crossing neurites (µm)
Smo localization dictates Hh signaling outcome

was quantified by staining for Isl1 and 2 (Isl1/2). Number of Isl1/2-positive cells per EB was counted. Shown are the data from two experiments, representing ≥6 EBs. (C, E, G) Detection of class III β-tubulin-positive neurites by immunofluorescence, quantification of the relative frequency of distance between crossing neurites in SmoΔ− ES cells transfected with vector, SmoWT, or SmoΔCLD. Frequency data represent >10 measurements for at least five EBs in three experiments. The average distance between crossing neurites in induced EBs was statistically different from the vector-transfected cells for SmoWT-transfected or SmoΔCLD-transfected cells, but was not for the noninduced EBs. ***P < 0.001 by Student’s t test; n.s., not significant. (D, F, H) Quantification of the number of Isl1/2-positive cells per EB formed from SmoΔ− ES cells transfected with vector, SmoWT, or SmoΔCLD. Shown are the data from two experiments, representing ≥6 EBs. (I) Cells were transfected with SmoΔCLD and EBs were formed in the presence of RA and SAG, and in the presence or absence of 0.5 µM MK-886. After 4d, EBs were stained for Isl1/2 and class III β-tubulin. Shown are the data from 2 experiments, representing five EBs in each experiment. Frequency data represent 10 measurements per EB.

DISCUSSION

Here, we describe the impact of subcellular localization of Smo on its signaling ability and unravel a negative correlation between its ciliary localization and its chemotactic signaling capacity. The observation that mutations in Smo that cause it to no longer localize at the primary cilium, or the absence of proteins necessary for formation or function of the primary cilium, caused an enhanced chemotactic response indicates that ciliary localization of Smo is not required for chemotactic signaling through Smo.

Although the cilium can be enriched for Smo, it is unlikely to contain the majority of Smo present in a cell. Thus, the enhancement of chemotactic signaling by defective localization of Smo to the primary cilium cannot be explained by the hypothesis that, in wild-type cells, Smo resides in the cilium and thus is unavailable for chemotactic signaling. Instead, it is likely that the consequences of Smo activation are dependent on its subcellular localization. Whereas the trafficking events culminating in accumulation of activated Smo to the primary cilium favor the Gli-mediated transcriptional response, localization of activated Smo to other sites in a cell results in a chemotactic response involving local rearrangement of the cytoskeleton.

Our data indicate that Shh chemotactic signaling does not require trafficking of Smo to the primary cilium. This is expected because pathway components, when concentrated in a single cellular structure like the primary cilium, cannot easily sense Shh concentration differences along the cell surface. Although the migratory response to PDGF depends on the primary cilium [39], we propose that Shh-mediated chemotaxis involves detection of the Shh signal by Smo receptors distributed over a larger domain of the cell membrane. Furthermore, Shh signaling to the downstream transcriptional mediators is dispensable for Shh-stimulated chemotaxis [24]. Transcriptional events in the nucleus lack the directional information needed for chemotaxis and does not occur on a timescale compatible with that of Shh-stimulated chemotaxis. Thus, several lines of evidence are consistent with Smo-mediated chemotactic signaling being independent of the primary cilium.

The increased chemotactic signaling capacity of Kif3A− MEFs and cells transfected with SmoΔCLD correlated with differential Smo glycosylation at the cell surface, suggesting that the different itineraries of Smo separated in the late Golgi or soon thereafter. Because SmoΔCLD and SmoC151Y were detected at the cell surface, where it is predicted to
mediate Shh chemotaxis, only in the partially glycosylated form, full glycosylation of Smo may not be strictly required for chemotactic signaling.

Genetic loss of the primary cilium, as well as forms of Smo that show impaired localization to or retention in the cilium, compromises the transcriptional response to Shh. However, in all these conditions the chemotactic response remains intact. The evolutionary conservation of the chemotactic response (fruit fly or zebrafish Smo triggered chemotactic signaling, but not transcriptional signaling in mouse cells) also argues that chemotaxis may be a more general Shh response, whereas the transcriptional response may be more divergent. Whether Shh-mediated chemotaxis or pathfinding is uncoupled from the transcriptional response in vivo and the importance of Shh-stimulated chemotaxis mediated by Smo outside the primary cilium in development remain to be determined.

A role for primary cilia also occurs for the related Wnt pathway. In contrast to Shh signaling, for Wnt signaling, the absence of a primary cilium favors signaling through a transcriptional pathway, one involving β-catenin [44]. Although the mechanisms underlying the cilia-dependent regulation of transcriptional activation of the Wnt pathway are presumably very different from the one that we described for Shh, it is interesting to see how separate developmental pathways use the primary cilium as a determinant for qualitative differences in the responses to their inducers.

**MATERIALS AND METHODS**

**Materials**

Cycloheximide, chloroquine, actinomycin D, pertussis toxin, MK-886, PP1, PP2, and MK571 were from Sigma (St. Louis, MO). Cyclopamine was from Biomol (Plymouth Meeting, PA). Purmorphamine and SAG were from EMD Biochemicals (Darmstadt, Germany). Cell Tracker Green CMFDA (5-chloromethylfluorescein diacetate) was from Invitrogen (Carlsbad, CA). Recombinant ShhN was from R&D Systems (Minneapolis, MN).

**Constructs**

The SmoWT, SmoΔCLD, SmoC151Y in pCS107 constructs were a kind gift from J. Reiter [14]. SMOM2 and SMOM1 were from Genentech (F. de Sauvage, South San Francisco, CA). pcDNA3.1 vector was obtained from Invitrogen. The Gli-luciferase reporter and the Renilla control were a kind gift from H. Sasaki [35]. pEGFP-N1 and the nuclear factor κB (NFκB) reporter were from Clontech (Mountain View, CA). Zebrafish Smo in pCS107 was a gift from J. Chen. Fruit fly Smo in pAC (S. Ogden) was cloned into pCS107 using HindIII and Xhol.

**Cell culture**

All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen). Shh-LIGHT II cells (ATCC) [28] were grown in medium supplemented with 400 µg/ml neomycin and 150 µg/ml zeocin.
Transfections
All transfections were performed using Effectene (Qiagen, Hilden, Germany). For transfections in 6-well plates, 2 µg DNA was used at a 1:15 ratio of DNA:Effectene. For 12-well plates, 1 µg DNA was used. Cells were incubated with transfection complexes for 16 hours.

Luciferase assay
Cells grown to 70% confluence in a 12-wells plate were transfected as described above with a Firefly Gli-reporter construct and a CMV-driven Renilla luciferase control. Cells were grown to confluence in medium with 10% FCS, and medium with 0.5% FCS was added 24 hours prior to the experiment. After stimulation, luciferase activity was measured using the Dual-Glo Luciferase Assay System from Promega (Madison, WI). Raw relative luminescence units (RLU) were corrected for their Renilla control.

Modified Boyden chamber migration assay
Cell migration assays were performed as described [16]. Cells were labeled with 10 µM CellTracker Green (Invitrogen) according to manufacturer’s protocol. After labeling, cells were detached with 5 mM EDTA, and cells were transferred into FluoroBlok Transwell inserts (BD Falcon) at 5 x 10^4 cells per insert. Chemoattractants were added to the bottom compartments of the Transwell dishes. GFP-spectrum fluorescence in the bottom compartment was measured in a Victor3 plate reader (PerkinElmer, Waltham, MA) every 2 min for 99 cycles (approximately 3 hours). First, background fluorescence (medium without cells) was subtracted from all values. Next a control with cells but no attractant was subtracted each time point, this yields net migration to the different attractants. Starting points of migration were set to 0. For Smo^-/- MEFs, each RFU represented ~5 cells. Using this conversion of 5 cells per RFU, we calculated the number of migrated cells as 28% of the total number of cells for the SmoWT-transfected MEFs using purmorphamine as the attractant, whereas 16% of the total number of cells migrated to the bottom well in the absence of attractant.

Fluorescence microscopy
Cells grown on cover slips were washed with PBS and fixed with 4% formaldehyde in PBS for 20 min. The tubulin cytoskeleton was stained with a 1:1000 dilution of an antibody recognizing acetylated α-tubulin (Sigma) dissolved in 10% normal goat serum in phosphate buffered saline with 0.1% Triton X-100. For transfected Smo, we used the antibody 9B11 that recognizes Myc (Cell Signaling) at 1:5000. SMOM2 was visualized using anti-gD (clone ID3) at 1:100. We used an antibody recognizing Rab-11 (BD Biosciences) at 1:500 and the antibody ID4B that recognizes LAMP1 (Developmental Studies Hybridoma Bank, Iowa) at 1:100. Appropriate secondary antibodies were used at 1:500 (Invitrogen). After staining, cells were mounted in ProLong Antifade mounting medium (Invitrogen) and examined on a Zeiss Observer Z1 epifluorescence microscope (Wetzlar, Germany). Colocalization was analyzed in ImageJ.
**Western blotting**

Cells were lysed in Leammli buffer and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes, blocked with 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBS-T), and incubated in the 9B11 antibody diluted 1:2000, the antibody recognizing β-tubulin diluted 1:2000, or the antibody recognizing GFP diluted 1:1000. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies were used at 1:5000. Proteins were visualized with a FujiFilm LAS 3000 imager.

**Embryoid body neuralization**

EB differentiation was performed according to Wichterle et al. [42]. EBs were stained with 1:500 Tuj1 primary antibody (Covance, Princeton, NJ) or an antibody recognizing Isl1/2 antibody (Covance) diluted 1:1000 and imaged with a fluorescence microscope. Network density was quantified as described [25]. Neurite length between crossing neurites (nodes) was measured in Zeiss Axiovision software. Frequency distribution analysis of the measured lengths was analyzed using Graphpad Prism.

**Surface biotinylation**

Cells in 100-mm dishes were transfected with 1 µg pEGFP-N1 and 3 µg SmoWT, SmoΔCLD, or SmoC151Y (all in pCS107). Following surface biotinylation and lysis according to the manufacturer’s protocol (Cell Surface Protein Isolation Kit; Pierce, Rockford, IL), a fraction of the lysate was immunoblotted for GFP to correct for transfection efficiency. The remaining lysate was precipitated on a streptavidin column according to the manufacturer’s instructions. Precipitated proteins were immunoblotted for Smo. Lysates that were treated with Endo H were subjected to 1500 units of Endo H (New England Biolabs, Ipswich, MA) for 1 hour at 37°C prior to Western blot analysis.

**Statistical analysis**

Statistical analyses were performed with GraphPad Prism 4.0.

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**Author contributions**

M.F.B. and H.D. performed the experiments and analyzed the data. M.F.B., H.D. and H.R. designed the experiments. M.F.B. and H.R. wrote the paper. Competing interests: The authors have no conflict of interest to declare.
REFERENCES


SUPPLEMENTARY MATERIALS

Figure S1. Smo mutants fail to localize to the primary cilium.

Figure S2. Rab11 functions in the trafficking of Smo to the primary cilium.

Figure S3. Activating mutations in Smo do not rescue the transcriptional response phenotype or ciliary localization defect of SmoΔCLD.

Figure S4. Activation of non-ciliary Smo results in activation of Src.

Figure S5. SmoWT and SmoΔCLD are expressed at similar levels following transfection of Smo+/− ES cells.

Figure S1. Smo mutants fail to localize to the primary cilium. (A-D) Smo−/− MEFs were transfected with mutant forms of Smo and localization of Smo was visualized by immunofluorescence for the Myc tag on Smo or the gD tag on SMOM2. Primary cilia were visualized by staining for acetylated α-tubulin or Arl13B. Scale bar, 20 µm. (E) The abundance of Smo was assessed by Western blot. Both a short and long exposure of the blot are shown. (F) The fraction of cells with primary cilium (gray bars) was determined by staining for acetylated α-tubulin. Ciliary localization of Smo following DMSO control or 2 µM purmorphamine for 16h was quantified by staining for Smo. Data are the mean ± SEM of 50 cells measured in 3 experiments. Statistically significant differences compared to SmoWT are indicated, *P < 0.05 by Mann-Whitney statistical test.
Figure S2. Rab11 functions in the trafficking of Smo to the primary cilium. (A) Smo−/− MEFs were transduced with control pLKO.1 virus or TRC clone 100344 shRNA against Rab11, target sequence; CAGAGATATACCGCATTGTTT. Two weeks after transduction and selection with 1µg/mL puromycin, quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for Rab11 and Gapdh was performed to assess knockdown. (B) Two and 7 weeks after transduction, cells were lysed and Western blot analysis for the abundance of extracellular-regulated kinase 1 and 2 (ERK1/2) (Cell Signaling Technologies) and Rab11 (BD Biosciences) was performed. Dashed line indicates part of membrane not shown. Bands shown are from the same membrane and exposure. (C) Stable knockdown cells were transfected with SmoWT, and ciliary localization was assessed by colocalization with acetylated α-tubulin, following water control or 500 nM SAG treatment for 4 hours. Quantification of at least 50 cells over 3 experiments is shown, mean ± SEM. *P < 0.05, ***P < 0.005 by Mann-Whitney statistical test.

Figure S3. Activating mutations in Smo do not rescue the transcriptional response phenotype or ciliary localization defect of SmoΔCLD. (A) Amino acid sequence alignment of human SMO, activated SMOM2, and indicated mouse forms of Smo generated by site-directed mutagenesis (SmoCLD-A1). The SMOM2 mutation is marked with an asterisk. (B-D) Smo−/− MEFs were transfected with indicated forms of Smo and immunofluorescence was performed for Smo. (E) Smo−/− MEFs were transfected with together with a Gli-luciferase reporter construct. Activation of the transcriptional response pathway was measured and is shown as mean fraction of wild-type mSmo (set to 1), ± SEM, n=3. (F) Smo−/− MEFs were transfected and chemotaxis to 2 µM of purmorphamine was assessed. Data are shown as the mean migration ± SEM, n≥3. Statistically significant differences compared to wild-type mSmo are indicated in panels E and F. *P < 0.05; ***P < 0.005, by Student’s t statistical test.
Smo localization dictates Hh signaling outcome

Figure S4. Activation of non-ciliary Smo results in activation of Src. Smo−/− MEFs were transfected with SmoWT or SmoΔCLD, serum starved, and treated with 2 µM purmorphamine or DMSO control for 10 min. Cells were subsequently lysed in Laemmli buffer and Western blotting was performed with an antibody recognizing phosphorylated Src (Cell Signaling Technologies) at 1:1000 or an antibody recognizing β-actin (Santa Cruz Biotechnologies) at 1:2000. Blot shown is representative of 3 experiments.

Figure S5. SmoWT and SmoΔCLD are expressed at similar levels following transfection of Smo−/− ES cells. Smo−/− ES cells were transfected with vector, SmoWT or SmoΔCLD. After 48 hours, cells were subsequently lysed in RIPA buffer, and protein levels were measured by BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were mixed with Laemmli buffer and Western blotting was performed with an antibody recognizing the Myc tag on Smo. Shown is a blot from a single experiment.