Novel Mechanisms and Functions for Hedgehog Signaling in Development and Disease
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Sonic Hedgehog Induces Transcription-Independent Cytoskeletal Rearrangement and Migration Regulated by Arachidonate Metabolites

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SUMMARY
Sonic hedgehog (Shh) is a morphogen pivotal for development and tissue maintenance. Biological effects of Shh are mediated through a pathway that involves binding to Patched1 (Ptch1), thereby releasing Smoothened (Smo) from inhibition resulting in the activation of Gli transcription factors, which mediate the induction of Shh target genes. Here, we describe a novel signal transduction pathway for Shh, which is transcription/translation-independent, SuFu insensitive, and consequently independent of Gli-mediated induction of transcription. Through this alternative pathway Shh, transduced via Smo, induced altered cell morphology together with lamellipodia formation. Migration assays demonstrate that this cytoskeletal rearrangement mediates the migratory response to Shh. This Shh-induced, Smo mediated migration utilizes and requires the metabolism of arachidonic acid through the 5-lipoxygenase pathway. These data provide a link between a seemingly novel Gli-independent Hh signaling pathway and the leukotriene metabolism, and might explain the developmental abnormalities observed in both patients with defective leukotriene metabolism as well as in rodent models of defective Rho family GTPase signaling.

INTRODUCTION
Hedgehog (Hh) proteins constitute a highly conserved family of intercellular signaling molecules (Bijlsma et al., 2006a; Lum and Beachy, 2004; McMahon et al., 2003). Originally identified as a Drosophila segment polarity gene required for embryonic patterning, several mammalian homologues have now been discovered; Indian (Ihh), Desert (Dhh) and Sonic Hedgehog (Shh), the latter being the most extensively characterized. These Hh proteins are fundamental regulators of embryonic development, as illustrated by dramatic embryonic malformations seen in humans and mice with perturbed Hh signal transduction. Equally important are the consequences of inappropriate activation of the Shh response in tumor formation. For instance, even one-allelic loss of the Hh receptor Patched1 (Ptc1) causes Gorlin syndrome, which is characterized by frequent formation of basal cell carcinomas (Gorlin, 2004). Besides being causative in basal cell carcinoma of the skin, activation of the Shh response is also involved in the development of endodermal cancers (Beachy et al., 2004; Benson et al., 2004; Toftgard, 2000; Watkins and Peacock, 2004). In addition, the Hh pathway remains active in the post-embryonic period, maintaining tissue integrity in a variety of tissues including the gastrointestinal tract and the immune system (Benson et al., 2004; van den Brink et al., 2004; van den Brink et al., 2001) as well as revascularization after ischemic stress (Kusano et al., 2005; Pola et al., 2001). Despite the obvious importance of Hh signaling for human (patho)physiology the molecular details underlying this signaling cascade remain not completely understood.

Hh biogenesis and signal transduction are unusual, many features appear unique to this signaling system. After synthesis, Hh undergoes autocatalytic cleavage followed by lipid modifications resulting in a secreted protein that is both sterolated and palmitoylated (Pepinsky et al., 1998; Porter et al., 1996). Its receptor Ptch1 is remarkable, as it does not
convey the Hh signal to the intracellular components of the pathway itself like a conventional receptor. Rather, binding of Hh to Ptc1 alleviates the inhibitory effect of Ptc1 on another membrane receptor, Smoothened (Smo), which, in the absence of this repression, activates target gene transcription. Most likely, Ptc1 utilizes vitamin D3 or a similar molecule to inhibit Smo (Bijlsma et al., 2006b). The alleviation of Smo inhibition is preceded by internalization of Ptc1 and Smo following binding of Hh (Denef et al., 2000; Incardona et al., 2002). In mammals, separating Smo from Ptc1-dependent inhibition results in the activation of the Gli transcription factors (Alcedo et al., 1996; Chen and Struhl, 1998; Ingham et al., 1996; Marigo et al., 1996; van den Heuvel and Ingham, 1996) through a process which is still largely unknown but involves the dissociation of Gli from a complex that presumably involves, Kif3/Costal2 and Fused/Suppressor of Fused (SuFu) (Dunaeva et al., 2003). Although the exact actions of the different Gli proteins and their processing with respect to gene activation and repression remain subject to debate, together, this Gli-dependent signaling cascade may be termed the classical Hh pathway. Note that for the sake of simplicity, we will refer to the 3 Gli proteins as Gli in this manuscript.

Many features of Hh signaling are similar to Wnt signaling which, like Hh, is a fundamental regulator of vertebrate morphogenesis. In canonical Wnt signaling (Reya and Clevers, 2005), the ligand Wnt binds to Frizzled (a serpentine protein similar to Smo) and reminiscent of the Hh pathway, activation of the receptor prevents degradation of a central transcriptional mediator, β-catenin in the case of Wnt signaling. For Wnt signaling, however, in addition to this canonical pathway, also a non-canonical signaling pathway, signaling though Frizzeld, but not via β-catenin, has been identified that is essential for a plethora of Wnt functions in pathophysiology (Kuhl et al., 2000). This consideration prompted us to investigate whether, in analogy to Wnt signaling, Hh signal transduction also has alternative pathways that do not directly involve activation of transcription. Suggestive data for such signaling come from neuronal development. It has emerged that Hh is an important chemotactic factor, with respect to axonal guidance during development of the central nervous system (Charron et al., 2003), an action that fits poorly with Hh only acting through Gli-dependent transcription. Thus, we investigated the effects of Hh on the actin cytoskeleton of mouse mesenchymal fibroblasts, as the actin cytoskeleton is a highly sensitive readout for a variety of signal transduction events (Ridley et al., 2003). Hereby we identified a novel pathway in response to Shh, mediating rapid actin reorganization, dependent on arachidonic acid metabolism through the 5-lipoxygenase pathway and functionally significant as an inducer of cellular migration.

**RESULTS**

**KINETICS OF THE TRANSCRIPTIONAL RESPONSE TO SHH**

C3H/10T1/2 mouse mesenchymal fibroblasts are responsive to Hh signaling. When transfected with the δ51-LucII Gli-reporter (Sasaki et al., 1997) and employing CMV-driven Renilla luciferase as a control, we observed activation of the reporter in response to 6h stimulation with 1 µg/ml Shh (Figure 1A). The stimulation could be blocked by the addition of 1 µg/ml Shh blocking antibody 5E1 (Ericson et al., 1996). Also shown in panel 1A, stimulating the C3H/10T1/2 cells with Shh for 3 days resulted in a more pronounced response. Importantly, we did not observe Gli-activation after a 10 min Shh stimulus. Using Ptc1−/− MEFs that express β-galactosidase in response to Shh-stimulus, no response to 1 µg/ml Shh was seen 10 min after addition of the ligand (0.37% ± 8.4 increase in pathway activity, not shown in Figure 1) whereas a 45% increase in reporter activity was observed after 16h (Goodrich et al., 1997).

**FIGURE 1. KINETICS OF TRANSCRIPTIONAL SHH RESPONSE**

(A) Gli-reporter transfected cells were treated with recombinant N-terminal Shh (1 µg/ml) for 10 min, 6h or 24h (22, 16 and 24h post-transfection respectively). A co-stimulation with 1 µg/ml Shh and 1 µg/ml 5E1 Shh-blocking antibody for 6h was included as a control. (B) Cotransfection of various Hh-pathway components as used in this study to assess their functionality showed expected responses (lys1 24h post-transfection). Values shown are corrected for an internal CMV-Renilla luciferase standard. Depicted is the mean ±SEM; n ≥4; *p<0.05; **p<0.005; One-tailed Unpaired t test. RLU = relative light units.
To assess the functionality of the expression constructs employed in this study, we co-transfected these vectors with the reporter construct and assayed reporter activity. The Gli-inhibitory protein SuFu abolished Gli activity in the presence of overexpressed Gli1, testimony of the low basal activation state of the Hh pathway in the cells used (Figure 1B). Not unexpectedly, Gli1 overexpression itself was markedly stimulatory. Together these results show that the transcriptional response to Shh occurs happens in a timeframe measured in hours, not minutes, consistent with the expected time necessary for receptor trafficking, Gli activation, and transcriptional activation of genes and translation of proteins induced by Shh.

**Shh induces cytoskeletal rearrangement**

After establishing the timeframe of transcriptional Hh responsiveness of our model system, we investigated whether this cell type might display cytoskeletal reorganization in response to Shh. It appears that a 10 min stimulation with 1 μg/ml Shh resulted in a significant reorganization of the actin cytoskeleton as assessed by phalloidin-TRITC staining (Figure 2A). Especially, large flat sheet-like structures (indicated by arrows), most likely lamellipodia, were formed in response to Shh as compared to untreated cells. Furthermore, in contrast to the elongated appearance of control cells, Shh-

**Figure 2. Shh induces cytoskeletal rearrangements**

(A) Mouse mesenchymal fibroblasts were serum starved for 6h and stimulated with either solvent control or 1 μg/ml Shh for 10 min. Staining of the actin cytoskeleton with TRITC conjugated phalloidin showed the appearance of large sheet-like structures on the cell’s edges as indicated by the arrows. Pretreatment with cyclopamine abolished this response (pretreatment 10 min prior to Shh addition with 10 μM cyclopamine). Scale bar = 100 μm.

(B) Larger magnification of control- and Shh stimulated cells. Again, arrows indicate lamellipodia. Scale bar = 10 μm.

(C) Deconvolved close-up images of the lamellipodia formed in response to Shh show distinct ruffling of the trailing edge of the cells.

(D) Cells were stimulated and stained as for panel A and subsequently the number of cells with lamellipodia (as indicated by arrows in B) was quantified. For SuFu overexpression experiments, cells were transfected with either control vector or SuFu expression vector 16h prior to stimulation (depicted is the mean ±SEM; n=100 in 3 independent experiments; ***p<0.005; One-tailed Unpaired t-test).
stimulated cells appeared less angular. Cyclopamine pretreatment prevented Shh-induced cytoskeletal rearrangements demonstrating these changes require Smo activation. Larger magnification images of experiments as shown in Figure 2A are shown in Figure 2B, focusing on the lamellipodia formed in response to Shh (indicated by arrows). Shown in Figure 2C are detailed, deconvolved images of the Shh-induced lamellipodia, illustrating the distinct ruffling of the cells, caused by lamellipodia that detach from the substrate and retract into the cell. A known function of these lamellipodia is in cell motility, in which a lamellipodium adheres to the substrate in the direction of cell movement, forming the leading edge of a cell on its track.

A single-blind quantification and statistical justification of the abovementioned Shh-dependent and cyclopamine-sensitive lamellipodia formation is presented in Figure 2D. As at the 10 min. time point employed for these experiments no activation of the Gli-reporter could be detected (Figure 1A), the actin reorganization observed most likely does not involve Gli activation. This notion is further confirmed in experiments in which the inhibitor of canonical Hh signaling SuFu was overexpressed. Transfection of SuFu did not alter the amount of Shh-induced lamellipodia (Figure 2D), emphasizing the Gli-independent nature of this response. We conclude that in this model system, Shh is capable of inducing a Smo-dependent but Gli-independent reorganization of the actin cytoskeleton within minutes.

**SHH ACTS A CHEMOTRACTANT**

Lamellipodium induction is associated with enhanced cell motility (Mitchison and Cramer, 1996; Ridley et al., 1999). Hence, to assess the functional relevance of the Shh-induced cytoskeletal alterations, migration assays were performed using 2 μg/ml Shh as chemoattractant. We employ higher Shh concentrations in the Transwell system (See Figure 3A for the migration assay procedure) than in the actin reorganization experiments because the Transwell system prevents rapid diffusion of Shh to the cells resulting in a lower effective concentration (0.13 μg/ml Shh after 5 min in top well, 0.42 μg/ml Shh at 10 min and 0.65 μg/ml Shh after 1h). Detached cells were resuspended in serum-free medium and introduced in the Transwell system. As cells move through the membrane towards the Shh gradient, the fluorescence signal at the bottom side of the Transwell was determined as measurement of cell migration. The Fluoroblok membrane blocks any excitation light from cells in the top well. The fluorescent signal at the bottom side increased over time as more cells migrated through the membrane. In parallel to the experimental attractant, migration to 20% FCS as well as a no-chemoattractant control to subtract from the experimental conditions was included. Using a no-attractant control (that does show baseline migration), allows correction for aspecific effects of cell treatment or transfection.

As shown in Figure 3B, the chemoattractant capacity of 2 μg/ml Shh for mesenchymal fibroblasts compared well to that of 20% FCS. These data expressed as bar graph, as well as controls addressing the specificity of the response, are shown in Figure 3C. Migration to Shh was blocked by the addition of 2 μg/ml Shh blocking antibody 5E1, making contaminants in the recombinant Shh preparation an unlikely factor in this response. The specificity of the migratory effects observed as a novel Gli-independent response to Shh would require that the classical Pch1/Smo receptor pair mediates this effect; otherwise relatively aspecific interaction with other signaling systems might be involved. Importantly, however, the specific Smo-inhibitor cyclopamine abolished the migration to Shh. Furthermore, 100 nM Smo agonist (SAG) was an efficient chemoattractant indicating that Smo activation is sufficient for mediating migration.

To establish whether this response reflects directed migration towards a gradient of Shh, or is the result of a general increase in cell motility conferred by Shh, we set up an experiment in which we added 2 μg/ml Shh to both sides of the Transwell, i.e. to the top as well as the bottom well. As can be seen from Figure 3D, the absence of a Shh gradient completely abolished cell movement to the bottom face of the Transwell membrane, suggesting that the Shh response is indeed a chemotactic response and not dependent on enhanced chemokinesis.

Again, the timescale of these migratory effects appears to be inconsistent with a role for canonical transcription/translation-dependent Shh signaling. To formally confirm this notion, migration experiments with Shh were performed in the presence of actinomycin D (transcription inhibitor) or cycloheximide (translation inhibitor). Treatment with these inhibitors was efficient as both inhibitors completely abolished induction of Gli-reporter activity by Shh at decreased concentrations than used in the migration experiments (31%, -8% and -1% increase in pathway activity after Shh stimulation in control-, 0.5 μg/ml actinomycin D- and 0.1 μg/ml cycloheximide treated cells respectively; procedure as Figure 1A, 6h stimulation). As can be seen in Figure 3E, however, transcription or translation inhibitors did not inhibit migration. In agreement with these experiments, SuFu overexpression was equally incapable of diminishing migration to Shh (Figure 3C). Together these data demonstrate that the Shh-mediated actin reorganization is reflected in a migratory response to this morphogen and that this response is truly Gli-independent in its nature.
Cytoskeletal and migratory Shh responses are regulated by leukotriene synthesis

The cytoskeletal responses to Shh resemble EGF-induced cytoskeletal changes (e.g. (Ridley, 1995)), a response to EGF that requires the conversion of arachidonic acid into leukotrienes through the 5-lipoxygenase pathway (Peppelenbosch et al., 1993) and a similar requirement might be found for the cytoskeletal response to Shh.

Indeed, stimulation of serum starved fibroblasts with 1 mg/ml Shh resulted in the rapid intracellular synthesis of cysteinyl leukotrienes (Figure 4A), in accordance with the timeframe observed for the cytoskeletal changes in response to Shh. Therefore, we decided to assess the requirement of the 5-lipoxygenase metabolism in this Shh-mediated cytoskeletal reorganization and migration using specific inhibitors.

Cells were stimulated, stained and quantified as described for Figure 2 following preincubation with either 5 μM of the pan-lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) or 5 μM of the selective 5-lipoxygenase-activating protein (FLAP) inhibitor MK-886 (Dixon et al., 1990). Treatment with these inhibitors of lipoxygenases resulted in a significant reduction in the cytoskeletal response to Shh (Figure 4B). Artificial induction of leukotriene synthesis by addition
50 μM arachidonate did not increase the responsiveness to Shh in this read-out, but it did increase lamellipodium formation in control cells. Together, these data indicate that the Gli-independent response to Shh in this system is at least partly mediated through the induction of 5-lipoxygenase metabolism and that exogenously driven leukotriene production can cause cytoskeletal rearrangements similar to those found by Shh stimulation.

The importance of 5-lipoxygenase for functional Gli-independent Shh responses was confirmed in cell migration experiments. Migration to Shh was greatly reduced in cells pretreated with either NDGA or MK-886 (Figure 4C), the specific 5-lipoxygenase inhibitor MK-886 being the most effective. The fact that we find negative values after subtraction of the no-attractant control suggests that the NDGA- and MK-886 pretreated cells actually become less motile after...
Shh stimulation compared to cells not exposed to attractant. We explain this by the fact that the inhibition of leukotriene synthesis abolishes substrate competition for arachidonate between leukotriene and prostaglandin synthesis. Thus, in the presence of MK-886, Shh stimulation leads to an increase in prostaglandins that we suggest to inhibit cell motility in response to Shh (for an example of such a differential cytoskeletal response to leukotrienes or prostaglandins see (Peppelenbosch et al., 1993)). This suggestion was confirmed by the observation that inhibition of cyclooxygenases using indomethacin enhanced migration (Figure 4C). The 5-lipoxygenase specificity of the effects found was confirmed by using specific inhibitors of 12-lipoxygenase (baicalein 30 μM; 10 μM cinnamyl-3,4-dihydroxy-alpha-cyanocinnamate(CDC) which were unable to inhibit of Shh-induced migration. Arachidonate enhanced migration up a Shh gradient. Overall these data point to an intimate relationship between 5-lipoxygenase metabolism and the cytoskeletal rearrangements induced by Shh which seems reminiscent to that of growth factors such as EGF (Peppelenbosch et al., 1993).

**Lipoxygenase is not important for Gli-dependent Shh signal transduction**

Although we have shown the redundancy of Gli-mediated transcription in mediating the migratory response to Shh, this does not strictly rule out the involvement of the leukotriene metabolism in the Gli-dependent Hh pathway. In such a model, the effects observed are part of the classical cascade but do not need full downstream activation (i.e. Gli activation). To assess the requirement of the leukotriene metabolism in Gli mediated transcription, a luciferase assay as described for Figure 1A was employed. Reporter transfected cells were stimulated with 1 μg/ml Shh in the presence or absence of 5 μM MK-886, and after 6h cells are lysed and assayed. There was no difference in transcriptional response to Shh when 5-lipoxygenase synthesis was inhibited (Figure 4D). From these data we conclude that the leukotriene metabolism is not involved in Gli activation by the Hh pathway, but instead that it functions in a Hh-dependent response distinct from the one leading to transcriptional activation of Hh induced genes.

The specificity of the MK-886-mediated inhibition of migration towards Shh was assayed by performing a migration to FCS in the presence of MK-886 in parallel. Inhibition of 5-lipoxygenase synthesis by MK-886 specifically inhibited migration to Shh and not that to FCS (Figure 4E). Thus, the necessity of leukotriene metabolism is only required for migration towards Shh in our experimental paradigm.

**Discussion**

The Hh family of morphogens is generally assumed to act through a pathway leading to transcriptional activation and repression, involving members of the Gli transcription factor family. Studies, especially with regard to axonal guidance in the developing nervous system, have suggested that apart from this pathway, Hh also exerts developmentally relevant actions which due to the spatiotemporal character are unlikely to directly involve transcription. The data in the present study now indicate the presence of a Gli-independent Shh signaling pathway mediated by Smo that involves and requires the leukotriene metabolism to function in cytoskeletal rearrangement and cell migration to Shh. Although several novel receptors for Hh have been identified recently (McCarthy, 2002; Tenzen, 2006; Yao, 2006; Zhang, 2006), this is the first study describing a truly novel downstream transduction mechanism for Shh. This mechanism fits well with several of the known actions of Hh in pathophysiology: it has recently emerged that Hh is a critical mediator of revascularization in ischemic tissue (Bijlsma et al., 2006a) and directed vascular outgrowth is well known to involve highly regulated cellular migration processes. In this context, it is important to note that the 5-lipoxygenase metabolism is important for angiogenesis (Kanayasu et al., 1989) and that such 5-lipoxygenase metabolism emerged as an important effector of the Gli-independent cytoskeletal and migration effects observed in this study, although awaiting functional studies, this remains a correlation. Interestingly, defective 5-lipoxygenase metabolism is directly linked to developmental neuronal abnormalities (Mayatepek and Flock, 1998) reminiscent of those caused by a malfunctioning Hh pathway in human patients. This would imply that the 5-lipoxygenase-dependent Gli-independent branch of Hh signaling uncovered in this study is of substantial developmental importance. Indeed the recently described holoprosencephaly (HPE) phenotype caused by conditional Cdc42 deficiency (Chen et al., 2006) would support such a notion. Cdc42 regulates the actin cytoskeleton and affects the formation of filopodia and lamellipodia and Cdc42 activation is important for chemotaxis but not chemokinesis and its effect thus closely resembles the effects of Shh observed in this study. Furthermore, the Cdc42 knock out-dependent HPE has been described to be independent of transcriptional targets of Shh, indicating the involvement of a Gli-independent pathway. It is thus well possible that the effects described in the present study are an important constituent of improper Hh action in pathophysiology.

The stimulation of leukotriene synthesis by Shh seems linked to the induction of cytoskeletal alterations and induces cell migration. These are processes that are often linked to cancer cell invasiveness and metastasis, as well
as wound healing (Callahan et al., 2004; Karhadkar et al., 2004). Some of these processes require the metabolism of arachidonic acid through the 5-lipoxygenase pathway (Green et al., 2004; Muller et al., 2001) and particularly striking are the observations that both Hh signaling components and 5-lipoxygenase are highly expressed and required for the generation of tumors in the proximal gastrointestinal tract (Berman et al., 2003; Fan et al., 2004; Hennig et al., 2005; Hoque et al., 2005). The possibility that the Gli-independent signaling pathway as described in the present study indeed contributes to well-documented Shh effects in gastrointestinal cancer may thus warrant further investigation. Migration experiments using different cell types (not shown) demonstrated that the migration to Shh not to be unique the C3H/10T1/2 fibroblasts, as we found a migratory response to Shh for 3T3 fibroblasts, and various human cancer cell lines. Thus, Shh-dependent but Gli-independent Hh signaling leading to leukotriene production and subsequent migration may contribute to tumor dissemination of proximal GI tract cancers. As many other oncologically relevant stimuli (e.g., EGF) are capable of inducing leukotriene synthesis as well, however, the actual physiological relevance for Shh-dependent leukotriene synthesis in cancers of the proximal gastrointestinal tract remains uncertain; at any case Gli-dependent transcription seems the major mediator of Hh-dependent tumor growth in this compartment.

The identification of a novel Hh response pathway that is reminiscent of the non-canonical Wnt signaling pathway further strengthens the evolutionary similarity between these two important developmental pathways (Kalderon, 2002; Medina et al., 2000). An obvious difference, however, between non-canonical Wnt signaling and Gli-independent Hh signaling is that canonical and non-canonical Wnt signaling involve different Wnt ligands and receptors whereas Gli-dependent and -independent Hh signaling are induced, in the present model system, by the same ligands and receptors.

Together, our studies point to a novel Gli-independent Shh signaling pathway. Formally our studies only show that the Gli proteins do not function as transcription factors to regulate cytoskeletal rearrangements and migration. As Gli proteins are primarily cytoplasmic one could argue that non-transcriptional actions of the Gli proteins in the cytoplasm might be responsible for the observed effects. However, as such cytoplasmic effects of Gli have not been reported to date, we believe the novel Hh pathway is indeed Gli-independent. A finding that supports this notion comes from migration experiments in which we used cells from mice knockout for the Gli transcription factors (Gli1−/−, Gli2−/−, Gli3−/−, Gli1−/−2−/− and Gli2−/3−/−) (Lipinski et al., 2006) and found no reduction in migration to Shh. However, as none of these cells lack all of the Gli transcription factors, we feel these results are not entirely conclusive.

The main challenge now emanating from the present study is the identification of the signaling pathways responsible for the activation of leukotriene synthesis. The recent advent of kinome profiling technology that enables the generation of comprehensive descriptions of cellular kinase activity without the necessity of a priori assumptions as to the signaling pathways affected should greatly aid the identification of these pathways.

**MATERIALS AND METHODS**

**REAGENTS**

Recombinant N-terminal mouse Shh was purchased from R&D (Abingdon, UK). SAG was a kind gift of James Chen. Indomethacin, NDGA and phalloidin-TRITC were from Sigma (St. Louis, MO). Cyclopamine was from Biomol (Plymouth Meeting, PA). CellTracker Green CMFDA (5-chloromethylfluorescein diacetate) was obtained from Molecular Probes (Eugene, OR). Celecoxib was kindly provided by Pfizer (New York, NY). The Gli1 construct was a generous gift from Dr Ruiz I Altaba. The SuFu expression vector was given by Dr Rune Tøftgård. The pcDNA3.1 vectors used for expression of abovementioned genes were obtained from Invitrogen (Carlsbad, CA). The Gli-Luciferase reporter and the Renilla control were a kind gift from Dr H. Sasaki.

**CELL CULTURE**

C3H/10T1/2 clone 8 mouse mesenchymal fibroblasts (ATCC; Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Cambrex, East Rutherford, NJ) supplemented with 10% fetal calf serum (FCS, Cambrex). Ptch1 heterozygote MEFs were cultured in DMEM supplemented with non-essential amino acids and 15% FCS. Routine cell culturing procedures were used and cells were never allowed to reach confluence. For fluorescence microscopy, cells were grown on 15 mm glass cover slips in 24 wells plates to 20-40% confluence.

**TRANSFECTIONS**

All DNA transfections were carried out using Effectene from Qiagen (Hilden, Germany) and performed as indicated in the supplied protocol. For transfections in 6-wells plates, 2 μg DNA was used at a 1:15 ratio of DNA/Effectene. Cells were incubated with transfection complexes for 16h, after which fresh medium was added for another 6-8h preceding further experimentation. Transfection of siRNA was performed using RNAiFect (Qiagen) in 6-wells plates with the same nucleic acid concentration and transfection reagent ratio as for the DNA transfections.

**LUCIFERASE ASSAY**

Cells grown to 70% confluence in a 24-wells plate were transfected as described above with a Firefly Gli-reporter construct and a CMV-
driven Renilla Luciferase control, to a total of 1 μg of DNA/well. Cells were serum starved, and 1 μg/ml of Shh was added for 10 min, after which cells were lysed. In another experiment, cells were stimulated with Shh for 3d. 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. For the *Ptch1*−/− MEFS, β-galactosidase activity was assayed by stimulating the cells as for the Gli-luciferase transfected cells, and lysis in 0.2% Triton X-100, 100 mM sodium phosphate pH 7.8. Lysates were incubated in Galecton (Applied Biosystems) in 1 mM MgCl₂, 100 mM sodium phosphate pH 8.0 for 45 min. The luminescent migration was started by the addition of Emerald enhancer in 200 mM NaOH.

**CELL migration**

Cells were grown to 70% confluence in 6-wells plates and prior to experimentation labeled for 1h with 10 μM CellTracker Green in serum-free medium. The dye was fixed by 1h incubation in medium with 10% FCS, and subsequently cells were washed and detached with 2 mM ethylenediaminetetraacetic acid (EDTA) in PBS. After complete detachment, cells were resuspended in serum-free medium, pipetted through a 70 μM cell strainer (BD Falcon, Franklin Lakes, NJ) transferred to 8 μM pore size HTS FluoroBlok Cell Culture Inserts from BD Falcon which were inserted in fitting 24-wells plates in which various attractant-containing media were present. Promptly, fluorescence values representing the number of cells on the bottom side of the insert were read 4 times every 2 min on a Series 4000 CytoFluor Multi-Well Plate Reader (Perseptive Biosystems, Framingham, MA). The raw fluorescence data were corrected for background fluorescence and fading of the fluorophore. No-attractant controls were subtracted at each measured time point to correct for any effects not due to active migration to the chosen attractant. Migration start points were set to zero. Data shown in bar graphs are the mean fluorescence of all measured cycles from base-line to plateau migration.

**Fluorescence microscopy**

Cells grown on cover slips were washed with PBS and fixed with 3.7% formaldehyde for 20 min. After fixation, cells were permeabilized and blocked in PBS/0.1% Triton X-100 supplemented with 10% FCS for 1h. The actin cytoskeleton was stained with 10 μg/ml phalloidin-TRITC in PBS/0.1% Triton X-100 (PBS-T) supplemented with 1% FCS. After staining, the cells were washed and mounted in Mowiol/DABCO aqueous mounting medium and examined using a Leica DMRA (Wetzlar, Germany) epifluorescence microscope. Images were captured on a cooled charge-coupled camera (KK Series, Apogee, Auburn, CA) operated by ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

**Leukotriene EIA**

Cells were grown in 100 mm culture dishes, serum starved, and stimulated with Shh. Cells were lysed in 1 ml of methanol, after which the methanol was evaporated in a SpeedVac concentrator. After reconstitution in 100 μl of EIA buffer, cysteinyl leukotriene content was assayed according to manufacturer’s directions (Cysteinyl Leukotriene EIA Kit, Cayman Chemicals, Ann Arbor, MI).

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**References**


