

LETTERS

Vertebrate Smoothened functions at the primary cilium

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The unanticipated involvement of several intraflagellar transport proteins in the mammalian Hedgehog (Hh) pathway has hinted at a functional connection between cilia and Hh signal transduction^{1,2}. Here we show that mammalian Smoothened (Smo), a seven-transmembrane protein essential for Hh signalling³, is expressed on the primary cilium. This ciliary expression is regulated by Hh pathway activity; Sonic hedgehog or activating mutations in Smo promote ciliary localization, whereas the Smo antagonist cyclopamine inhibits ciliary localization. The translocation of Smo to primary cilia depends upon a conserved hydrophobic and basic residue sequence homologous to a domain previously shown to be required for the ciliary localization of seven-transmembrane proteins in *Caenorhabditis elegans*⁴. Mutation of this domain not only prevents ciliary localization but also eliminates Smo activity both in cultured cells and in zebrafish embryos. Thus, Hh-dependent translocation to cilia is essential for Smo activity, suggesting that Smo acts at the primary cilium.

Nearly all interphase vertebrate cells possess a primary cilium that extends into the extracellular environment⁵. Recent studies have pointed to a role for primary cilia as sensors capable of transmitting diverse types of information from the environment⁶. One example of this is the photoreceptor outer segment, a derivative of the primary cilium and the site at which light activates the seven-transmembrane (7TM) protein rhodopsin⁷. This connection between 7TM proteins and cilia seems to be an ancient one, as odorant receptors function on the cilia of *C. elegans* sensory neurons⁴.

Mutations in genes required for cilia formation cause defects in mouse neural tube patterning indicative of altered Hh signalling^{1,2}. The mechanism by which these genes participate in Hh signalling is unclear, but one possibility is that their involvement signifies a requirement for cilia in Hh signal transduction.

Hh signalling in *Drosophila*, zebrafish and mice is transduced through Smo, a 7TM protein³. To investigate the expression of endogenous Smo during mouse development we made use of two polyclonal anti-Smo antibodies. The antibodies are highly specific, as no protein is detected in *Smo*^{-/-} samples by immunofluorescence and western blot analyses (Supplementary Fig. 1). During early somite stages, Smo protein is modestly upregulated in the cells of the node (Fig. 1a), an organizer involved in several developmental processes including left-right axis determination and floor plate induction.

One structural characteristic that distinguishes ventral node cells from other cells of the early embryo is the presence of a primary cilium⁸. Examination of Smo localization in the nodes of early headfold, late headfold, one-somite, three-somite and five-somite-stage embryos revealed that Smo is located predominantly on

primary cilia (Fig. 1b, c). Although Smo does not localize uniformly along the entire length of cilia (Fig. 1d), average expression on cilia is greater than threefold higher than elsewhere on nodal cells.

The increased level of Smo protein expression specifically in the node is notable given that *Smo* messenger RNA is present throughout

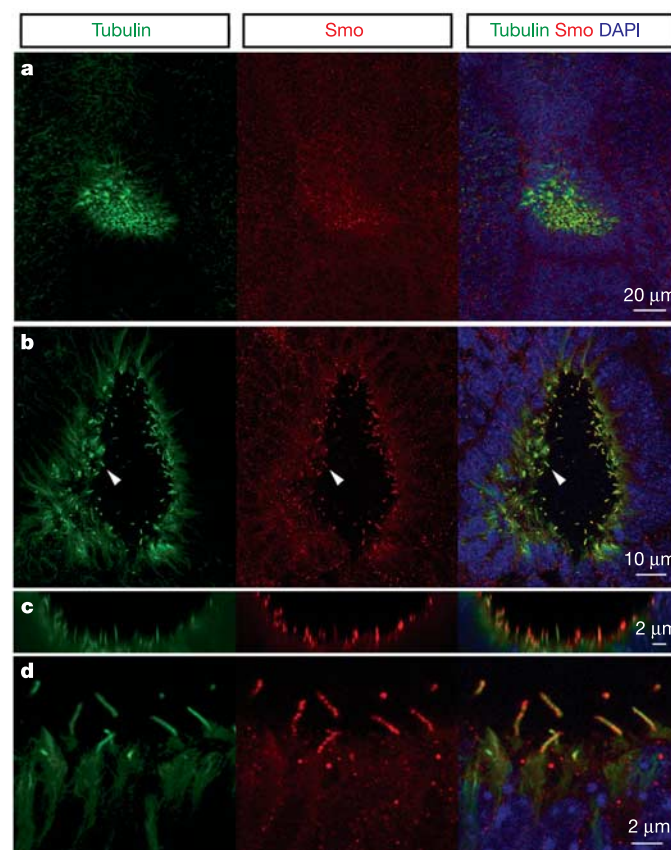


Figure 1 | Smo is expressed on nodal primary cilia. Expression of the ciliary marker acetylated Tubulin (green) and Smo (red). Nuclei are visualized with DAPI staining (blue). **a**, Ventral view of the node of a three-somite embryo with anterior oriented upwards. Elevated levels of acetylated Tubulin staining demarcate the ventral node cells. Smo expression in the node is approximately 170% that of surrounding cells. **b**, A higher magnification ventral view of a node demonstrating ciliary Smo co-localization with acetylated Tubulin (arrowhead). **c**, Optical cross-section through the node. **d**, High-magnification view of nodal primary cilia.

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the embryo⁹. Because *Sonic hedgehog* and *Indian hedgehog* genes (*Shh* and *Ihh*) are expressed in and near the node^{9,10}, respectively, we hypothesized that, as in *Drosophila*^{11,12}, mouse Smo might be stabilized in regions of Hh signalling. Indeed, Smo is upregulated specifically in and around the Shh-producing cells of the node, as demonstrated by the co-expression of Smo and Shh in early headfold-stage embryos (Supplementary Fig. 2).

To test whether Smo localization to cilia is regulated by Hh signalling, we created a renal epithelial MDCK (Madin–Darby canine kidney) cell line constitutively expressing Myc-tagged murine Smo. Smo is detectable on the plasma cell membrane and intracellular vesicles of MDCK cells (Fig. 2a), as previously described for *Drosophila* salivary gland and imaginal disc cells and rat KNRK (Kirsten

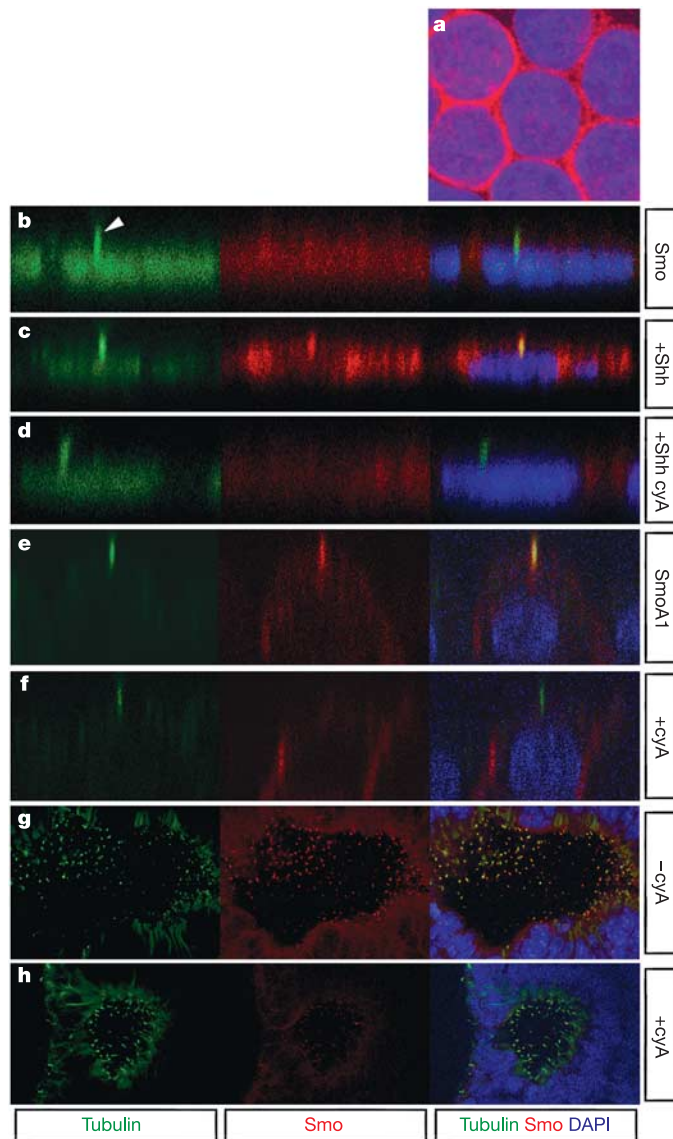


Figure 2 | Hh pathway activity regulates the ciliary localization of Smo. **a–f**, MDCK cell expression of acetylated tubulin (green) and the Myc tag of Smo (red). Nuclei are visualized with DAPI (blue). **a**, Transverse optical section of cells expressing wild-type Smo. **b–f**, Apical–basal optical sections. **b**, Cell expressing wild-type Smo. Acetylated tubulin expression marks the primary cilium (arrowhead). **c**, Smo-expressing cell cultured in the presence of Shh. **d**, Smo-expressing cell treated with both Shh and cyclopamine (cyA). **e**, Cell expressing SmoA1. **f**, SmoA1-expressing cell cultured in the presence of cyclopamine. **g, h**, Expression of acetylated tubulin (green) and Smo (red) in the nodes of headfold-stage embryos cultured in the absence (**g**) or presence (**h**) of cyclopamine. Nuclei are visualized with DAPI (blue).

murine sarcoma virus-transformed normal rat kidney) cells^{11–14}. We examined Smo distribution in MDCK cells cultured in the presence of Shh or cyclopamine, a Smo antagonist¹⁵. After culture for 1 h in Shh-conditioned medium, the amount of Smo present on the primary cilium is markedly upregulated (Fig. 2b). Similar localization of endogenous Smo is seen in Shh-treated mouse embryonic fibroblasts (MEFs) and IMCD-3 (inner medullary collecting duct) kidney cells (Supplementary Fig. 4). Addition of cyclopamine to Shh-conditioned medium eliminates detectable Smo from the primary cilium of MDCK cells (Fig. 2c).

The mouse homologue of an activated Smo mutant, SmoA1, has previously been demonstrated to affect Smo distribution¹⁴. Unlike wild-type Smo and unlike its behaviour in unciliated cells¹⁵, SmoA1 strongly localizes to the primary cilium of MDCK cells even in the absence of Shh (Fig. 2c). High concentrations of cyclopamine sufficient to prevent SmoA1 activation of the Hh pathway¹⁵ are able to eliminate SmoA1 from the cilium (Fig. 2d). To assess whether the presence of Smo on nodal cilia is also regulated by Hedgehog signalling, we cultured mouse embryos in cyclopamine. Comparison of the treated and untreated embryos reveals that cyclopamine markedly diminishes Smo localization to cilia (Fig. 2g, h), demonstrating that inhibition of Hh signalling *in vivo* correlates with the absence of Smo from the cilium.

The upregulation of Smo on the primary cilium in response to Shh or activating mutations suggests that the regulation of mammalian Smo trafficking may be more similar to that of *Drosophila* Smo than previously recognized. In both a *Drosophila* cell line and in imaginal discs, Hh induces the accumulation of Smo at the cell surface^{11,12}, whereas in rat KNRK cells, Shh stimulation induces the internalization of cell-surface Smo¹⁴. One reason KNRK cells process Smo differently may be that they lack cilia (J.F.R., data not shown). Our data suggest that Hh-dependent cell-surface enrichment of Smo may be common to both *Drosophila* cells and ciliated mammalian cells.

Ciliary localization of the *C. elegans* 7TM proteins ODR-10 and STR-1 depends on a hydrophobic and basic residue motif immediately carboxy-terminal to the seventh transmembrane segment¹⁶. This ciliary localization motif is also present in other 7TM proteins known to localize to mammalian primary cilia, such as somatostatin receptor 3 and serotonin receptor 6 (refs 17, 18). Sequence inspection reveals that Smo proteins also contain a hydrophobic and basic

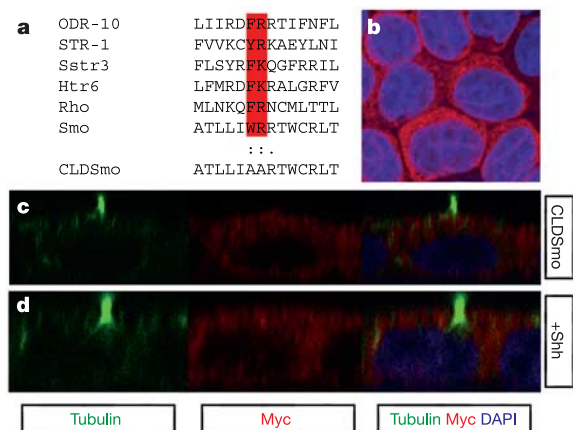


Figure 3 | Identification of a conserved Smo ciliary localization motif. **a**, Sequence comparison of the carboxy-terminal juxtamembrane sequences of 7TM proteins known to localize to cilia. ODR-10 and STR-1 are *C. elegans* 7TM receptors. Murine sequences are included for somatostatin receptor 3 (Sstr3), serotonin receptor 6 (Htr6), rhodopsin (Rho) and Smo. The conserved hydrophobic and basic residue motif is highlighted in red. CLDSmo is a mutant Smo lacking this motif. **b–d**, CLDSmo-expressing MDCK cells stained as in Fig. 2. **b**, Transverse optical section. Compare to Fig. 2a. **c**, Apical–basal optical section. **d**, Apical–basal optical section of a cell cultured in the presence of Shh.

residue motif carboxy-terminal to the seventh transmembrane segment (Fig. 3a).

To test whether this motif is required for ciliary localization, we replaced Trp 549 and Arg 550 of mouse Smo-Myc with alanines (Fig. 3a). The resultant mutant version of Smo is expressed on the cell plasma membrane and intracellular vesicles of MDCK cells, suggesting that it is folded and at least partly released from the endoplasmic reticulum (Fig. 3b). However, unlike wild-type Smo, this mutant Smo is absent from primary cilia, even in the presence of Shh (Fig. 3c, d). Consequently, we have named this mutant CLDSmo for ciliary localization defective.

These results indicate that the conserved motif carboxy-terminal to the last transmembrane segment of mouse Smo is a motif essential for ciliary localization, providing evidence that, like *C. elegans* 7TM proteins, mammalian 7TM proteins can utilize a juxtamembrane hydrophobic and basic residue sequence for proper localization to cilia. *Drosophila* Smo contains a similar motif, although cilia do not seem to be required for *Drosophila* Hh signal transduction^{19,20}. It is tempting to speculate that this domain may participate in the movement of *Drosophila* Smo to the plasma membrane in response to Hh signalling^{11,12}.

To test whether localization to primary cilia is required for Smo function, we compared the activities of wild-type Smo and CLDSmo in two established read-outs of Hh pathway activation. First, we determined Hh responsiveness in the presence of wild-type or CLDSmo using a Gli-dependent luciferase reporter in NIH-3T3 cells²¹. In the absence of transfected Smo, Shh-conditioned medium stimulates a threefold induction of normalized luciferase activity (Fig. 4a). Cells transfected with wild-type Smo exhibit a robust increase in luciferase activity, as previously reported²¹. In contrast,

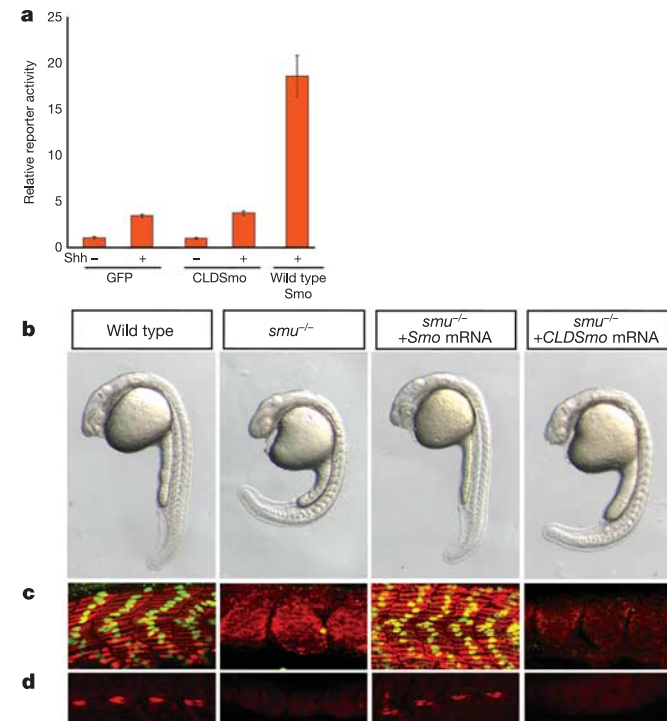


Figure 4 | CLDSmo is inactive. **a**, Induction of a Hh-responsive Gli-luciferase reporter in NIH-3T3 cells. Unlike wild-type Smo, CLDSmo expression fails to either activate the pathway or alter Hh responsiveness. Error bars indicate 1 s.d. **b**, Lateral views of 24 h post-fertilization zebrafish embryos. Shown are a wild-type control embryo and three *smu* mutants, two of which have been injected with 250 pg mRNA encoding either wild-type Smo or CLDSmo. **c**, F59 staining of slow muscle fibres (red), and Prox1-expressing superficial slow fibres (green). **d**, Engrailed (Eng) staining of muscle pioneers.

CLDSmo does not stimulate Hh pathway activity above control levels.

Second, we compared the function of wild-type Smo and CLDSmo in zebrafish. Hh signalling is required for specification of the zebrafish slow muscle lineage^{22–24}. Within the slow muscle lineage, Prox1-expressing superficial slow fibres (SSFs) require low to medium levels of Hh pathway activity, whereas Engrailed-expressing muscle pioneers require high levels of Hh pathway activity²⁵. Disruption of the zebrafish *Smo* homologue, *slow muscle omitted* (*smu*), prevents formation of both Prox1-positive SSFs and Engrailed-positive muscle pioneers, resulting in embryos that exhibit characteristic U-shaped somites (Fig. 4b). Injection of wild-type murine *Smo* mRNA into *smu* mutant embryos rescued this morphological defect and restored formation of both SSFs and muscle pioneers (Fig. 4b–d; see also Supplementary Table). In contrast, injection of CLDSmo mRNA into *smu* mutant embryos did not rescue shape, SSF formation or muscle pioneer formation (Fig. 4b–d; see also Supplementary Table). These results demonstrate that the ciliary localization motif is required for Smo function. Whether this motif might have roles in addition to ciliary localization remains to be determined.

Thus, not only does Smo ciliary localization depend upon Hh signalling, but Hh signalling depends upon a Smo ciliary localization motif. Taken together, these findings strongly suggest that Smo acts at the primary cilium to transduce Hh signalling and that Smo localization to cilia is a key regulated step in Hh pathway activation. Our studies provide an example of a 7TM protein translocating to the primary cilium in response to pathway activation, and it will be interesting to determine whether the localization of other ciliary 7TM proteins is similarly regulated.

Previous studies have suggested that redistribution of Smo to the appropriate subcellular location might be the main determinant of Smo activity¹³. Our findings indicate that in mammals, the primary cilium may be that subcellular location. However, whether Smo functions at the cilium in all cell types remains to be determined. In addition, how Smo activates the Hh pathway at the cilium remains unclear. One possibility is that Smo must translocate to the cilium to encounter an activator. An alternative possibility is that the cilium structurally coordinates Smo with downstream Hh pathway components to permit productive interactions. In support of this second possibility, β -arrestin 2, a component of the zebrafish Hh signal transduction apparatus and a known Smo interactor^{26,27}, is concentrated in cilia²⁸.

METHODS

Immunohistochemistry. Mouse embryos and MDCK cells were fixed with 4% PFA for 1 h at 4 °C, washed in PBT (PBS plus 0.2% Triton-X100), and blocked with PBT, 2% BSA and 1% goat serum for 1 h at 4 °C. Primary antibodies were added in block buffer and incubated overnight at 4 °C. Primary antibodies used in this study are anti-Myc (Abcam ab9106, 1:500), mouse anti-acetylated Tubulin (Sigma 6-11B-1, 1:1,000) and rabbit anti-Smo (Lifespan Biosciences A2666 and A2668, 1:200). The anti-Smo antibodies perform equivalently. Subsequently, cells were washed in PBT and incubated in a secondary block (PBT plus 10% donkey serum and 2% BSA) at room temperature for 1 h, followed by incubation with secondary antibodies (1:400 donkey anti-rabbit IgG-Alexa 594 and donkey anti-mouse IgG-Alexa 488; Molecular Probes) in PBT plus 2% BSA at room temperature for 1 h. After washes with PBT and 10 min 4',6-diamidino-2-phenylindole (DAPI) incubation, the samples were mounted and imaged with a Leica DMIRE2 confocal microscope. Images were processed using Leica Confocal, Metamorph 6.1 and Velocity 3.1 software. Zebrafish embryos were fixed overnight at 4 °C in 3% PFA, and staining was performed in PBT-BSA (PBS, 0.3% Triton X-100, 4% BSA, 0.02% NaN₃) using F59 anti-Myosin heavy chain (gift from F. Stockdale), anti-Engrailed (gift from S. Amacher) and anti-Prox1 antibodies (Chemicon International). Zebrafish embryos were visualized with a Zeiss LSM5 Pascal confocal microscope.

Expression constructs. Generation of the CLDSmo expression construct, pGE-CLDSmo-Myc-His, was engineered with the QuikChange II XL site-directed mutagenesis kit (Stratagene) using pGE-Smo-Myc-His (gift from P. Beachy) as template. The sequence of the pGE-CLDSmo-Myc-His open reading frame was confirmed.

Cell culture. MDCK cells were a gift from K. Mostov and were grown in MEM, 5% FBS, penicillin and streptomycin. To create MDCK lines stably expressing wild-type Smo or CLDSmo, the cells were transfected using Lipofectamine2000 (Invitrogen) and split 1:10 the following day. Beginning 36 h after transfection, cells were selected in 600 $\mu\text{g ml}^{-1}$ neomycin, and 5 days later, individual clones were isolated and expanded. The expression of wild-type Smo and CLDSmo was confirmed by western blot with goat anti-Myc antibody (Novus Biologicals). MDCK cell lines expressing comparable levels of Smo or CLDSmo were seeded at 3×10^5 cells per transwell filter (12 mm filter diameter/0.4 μm pore size, Costar) and fed daily for 6 days. Experimental samples were incubated in 10 μM cyclopamine, Shh-conditioned media, or both. Shh-conditioned media was generated as previously described²⁹.

Murine embryo culture. Embryonic day (E)8.0 embryos were isolated in HEPES-buffered DMEM plus 10% FBS, and then cultured in DR75 medium in the presence or absence of 10 μM cyclopamine. Embryos were cultured statically for 1 h at 37 °C in an incubator gassed with 5% CO₂ in air before being processed for immunohistochemistry as above.

Gli-luciferase reporter assay. NIH-3T3 cells were plated at 10⁵ cells per well in 24-well plates. On the next day, we transfected cells with *Renilla* luciferase (pRL-TK, Promega), Gli-luciferase reporter, and a Smo expression construct using Eugene6 (Roche) as previously described²¹. Two days later, cells were moved to low serum media (DMEM, 2% delipidated fetal calf serum, ITS supplement, 5 mM HEPES, penicillin and streptomycin) for 8 h, then treated with Shh-conditioned media for 18 h. All reporter assays were normalized for transfection efficiency using *Renilla* luciferase control (Dual luciferase assay, Promega).

Zebrafish mRNA injection. Embryos from *smu*^{b641} heterozygote intercrosses were injected at the one- or two-cell stage with 250 pg mRNA encoding either mouse wild-type Smo or CLDSmo, and raised at 28 °C until 24 h after fertilization.

Genotyping. Mouse *Smo* embryos were genotyped as described⁹. Zebrafish *smu*^{b641} embryos were genotyped by polymerase chain reaction (PCR) amplification (primers 5'-TTACGTCAACGCCTGTTCTTCCTT-3' and 5'-TCGCA-TAGTGTTCGCTTT-3'), followed by *DdeI* restriction enzyme digestion.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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