Rapid, direct activity assays for Smoothened reveal Hedgehog pathway regulation by membrane cholesterol and extracellular sodium

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Hedgehog signaling specifies tissue patterning and renewal, and pathway components are commonly mutated in certain malignancies. Although central to ensuring appropriate pathway activity in all Hedgehog-responsive cells, how the transporter-like receptor Patched1 regulates the seven-transmembrane protein Smoothened remains mysterious, partially due to limitations in existing tools and experimental systems. Here we employ direct, real-time, biochemical and physiology-based approaches to monitor Smoothened activity in cellular and in vitro contexts. Patched1–Smoothened coupling is rapid, dynamic, and can be recapitulated without cytoplasmic proteins or lipids. By reconstituting purified Smoothened in vitro, we show that cholesterol within the bilayer is sufficient for constitutive Smoothened activation. Cholesterol effects occur independently of the lipid-binding Smoothened extracellular domain, a region that is dispensable for Patched1–Smoothened coupling. Finally, we show that Patched1 specifically requires extracellular Na⁺ to regulate Smoothened in our assays, raising the possibility that a Na⁺ gradient provides the energy source for Patched1 catalytic activity. Our work suggests a hypothesis wherein Patched1, chemiosmotically driven by the transmembrane Na⁺ gradient common to metazoans, regulates Smoothened by shielding its heptahelical domain from cholesterol, or by providing an inhibitor that overrides this cholesterol activation.

Hedgehog signaling is critical in development and disease, but how cells respond to the secreted Hedgehog signal remains mysterious. A key step involves the regulation of the seven-transmembrane oncoprotein Smoothened by the 12-pass transporter-like Hedgehog receptor Patched1. We investigate the model that Patched1 is an ion-driven transporter of an endogenous lipidic Smoothened ligand. Whereas Patched–Smoothened regulation has traditionally been studied through indirect, downstream pathway readouts, we developed rapid, direct functional assays to dissect this step in simplified cell-based and in vitro systems. Cholesterol, a major membrane lipid, structurally activates purified Smoothened by engaging its membrane-spanning region. Patched1 activity depends on extracellular Na⁺ gradients, suggesting that transmembrane Na⁺ gradients, universal among metazoans, might power Patched1 transporter-like activity in Smoothened regulation.

Significance

The Hedgehog pathway is critical in development and disease, but how cells respond to the secreted Hedgehog signal remains mysterious. A key step involves the regulation of the seven-transmembrane oncoprotein Smoothened by the 12-pass transporter-like Hedgehog receptor Patched1. We investigate the model that Patched1 is an ion-driven transporter of an endogenous lipidic Smoothened ligand. Whereas Patched–Smoothened regulation has traditionally been studied through indirect, downstream pathway readouts, we developed rapid, direct functional assays to dissect this step in simplified cell-based and in vitro systems. Cholesterol, a major membrane lipid, structurally activates purified Smoothened by engaging its membrane-spanning region. Patched1 activity depends on extracellular Na⁺ gradients, suggesting that transmembrane Na⁺ gradients, universal among metazoans, might power Patched1 transporter-like activity in Smoothened regulation.


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proteins are functionally linked to sterols, including Niemann-Pick C1 (NPC1), a factor required for egress of LDL-derived cholesterol from lysosomes (18), and Dispatched, which helps to release the cholesterol-modified Hh ligand from Hh-producing cells (19, 20). Hh pathway activity is also highly sensitive to genetic or pharmacological blockade of cholesterol metabolism (21), an effect that maps downstream of Ptc1 and at or upstream of Smo. Supplying exogenous cholesterol to sterol-depleted cells reverses the resulting pathway inhibition (22–24). Furthermore, elevating cellular cholesterol to supraphysiologic levels can overcome Ptc1 inhibitory effects to promote Smo activity, as well as enhance the ability of Hh to derepress Ptc1 (23, 25). These findings raise the possibility that the endogenous Smo ligand might be cholesterol or one of its derivatives, and that Ptc1 might regulate Smo activity by controlling this sterol’s availability. Nevertheless, several fundamental questions remain unanswered. (i) Is Smo activity regulated by cholesterol itself or rather by one of its many metabolic products (such as oxysterols, bile acids, or steroid hormones), all of which are affected by the treatments described above? (ii) Does this sterol act directly on Smo or does it instead target an as yet unidentified pathway component between Ptc1 and Smo? (iii) Is cholesterol merely a cofactor required for efficient Smo activation by another lipid, or is it cholesterol sufficient to activate Smo in its own right? (iv) If cholesterol is a Smo ligand, where on Smo is it found in its point of action?

These remaining gaps in our understanding are difficult to resolve by the traditional approaches for measuring Hh pathway activity. Ptc1 and Smo function are typically evaluated using downstream transcriptional readouts or alterations in the ciliary accumulation of pathway components. Such indirect methods measure long-term consequences of changes in Ptc1 and Smo activity in ciliated cells, rendering them unsuitable for experimental manipulations that might disrupt ciliary trafficking or cause toxicity over an extended period. For example, alteration of cellular ion gradients fundamentally perturbs cellular physiology and may exert untoward effects on ciliary trafficking that would confound interpretation of these conventional downstream readouts. A rapid, direct assay of Ptc1 or Smo functional state might circumvent these issues, but such an approach has not been applied to study Ptc1 ionic requirements. Similarly, the outstanding questions regarding cholesterol regulation of Smo are unlikely to be addressed in complex, biochemically undefined environment of a living cell, where as yet unidentified sterols or proteins may contribute to Ptc1–Smo communication. Biochemical reconstitution, on the other hand, might rise to this challenge by enabling a stringent definition of the proteins, lipids, and other cellular factors that suffice to recapitulate Smo regulation in vitro. Unfortunately, existing methods to measure vertebrate Smo activity are indirect and require intact cells with primary cilia. This presents major obstacles for in vitro studies because it is currently not possible to reconstitute ciliary trafficking in cell-free settings. Thus, because conventional Hh pathway assays are long-term and indirect, Ptc1–Smo regulation has remained largely inaccessible.

In the present study, we investigate Smo regulation by developing a set of rapid, robust, and direct Smo activity sensors for cell-based and in vitro studies. We use these assays to re-capitulate Ptc1 effects on Smo outside the cillum, demonstrating that the underlying process is unexpectedly dynamic and can proceed in the absence of any cillum-specific proteins or metabolites. We also find that cholesterol is both necessary and sufficient for constitutive activation of purified Smo reconstituted in a defined lipid environment. This activation occurs independently of recently described interactions of cholesterol with the Smo cysteine-rich domain (CRD) (23, 26, 27), an extracellular region that we show is not required for Ptc1–Smo communication. Finally, we show that depletion of extracellular Na\(^+\) rapidly and reversibly extinguishes the effects of Ptc1 on Smo. This suggests that Ptc1 might use transmembrane Na\(^+\) gradients to power its catalytic cycle and provides evidence that Ptc1 may indeed function as a bona fide ion-driven transporter. Based on these data, we hypothesize that Ptc1 harnesses the energy stored in transmembrane Na\(^+\) gradients to either shield the Smo 7TM domain from cholesterol’s activating influence or provide an inhibitory factor that can block Smo even when cholesterol is present. Our study highlights the power of direct conformational sensors in simplified experimental systems to provide insights into Ptc1 and Smo regulation that lie beyond the reach of existing Hh pathway functional assays.

Results

A Cilium-Independent Smo Conformational Biosensor Based on G Protein Coupling. The cilium might provide a "privileged environment" for Ptc1–Smo regulation by harboring specialized protein and lipid factors that are required for this process. Alternatively, Ptc1–Smo regulation might proceed in the cilium, but the underlying mechanism might use factors that are distributed more ubiquitously throughout the cell, and the ciliary environment may be required mainly for downstream coupling to Gli transcription factors (Fig. S1A). One way to distinguish between these models is to test whether Ptc1–Smo regulation can be recapitulated in a nonciliary location. To this end, we designed a direct Smo conformational assay that takes advantage of Smo’s ability to couple to heterotrimeric G proteins in certain biological settings (28–31). G protein-based assays are ideal in this regard because activated 7TM proteins stimulate downstream coupling by directly binding a G protein heterotrimer, whose conformation changes almost instantaneously without the need for additional cellular components. While it remains controversial whether Smo employs G proteins to relay its “canonical” signal to Gli transcription factors (17, 32), we reasoned that G protein coupling could nevertheless provide a useful direct readout for Smo conformational state (Fig. S1B), allowing us to measure Smo regulation independently of ciliary trafficking.

We measured Smo G protein coupling using GloSensor (33), a firefly luciferase variant that emits light only in the presence of cAMP (Fig. 1A). We used HEK293 cells for these studies, as they contain minimal primary cilia (34) and are not transcriptionally responsive to Hh; this minimizes any potentially confounding influences of Smo trafficking or G protein coupling on Gli repression (17) in our experiments. To further ensure that our measurements do not depend on a ciliary pool of Smo, we made use of a direct fusion of a C-terminally truncated Smo to the inhibitory G protein Go\(_{i}\) (Smo–Go\(_{i}\)), thereby eliminating the cytoplasmic sequences required for Smo ciliary localization (35). We transfected the GloSensor cDNA into HEK293 cells, loaded the cells with a luciferase substrate, and raised cellular cAMP levels using the adenylyl cyclase (AC) agonist forskolin, leading rapidly to a large induction of luminescence (Fig. 1B). If Smo is able to couple to the fused inhibitory Go\(_{i}\) protein, we would expect Smo activity to reduce cAMP levels, leading to a decline in GloSensor luminescence. Indeed, cotransfection of Smo–Go\(_{i}\) with GloSensor decreased forskolin-induced luminescence, consistent with Smo–Go\(_{i}\) inhibition of AC (Fig. 1B). When we cotransfected Ptc1 and Smo–Go\(_{i}\) together with GloSensor, luminescence was restored to high levels, showing that Ptc1 inhibits Smo G protein coupling (Fig. 1B and Fig. S2A). Brief pretreatment (10 min) of Ptc1-expressing cells with the recombinant N-terminal fragment of Sonic hedgehog (ShhN) abolished the effect of Ptc1 on Smo–Go\(_{i}\) (Fig. 1B), confirming that our assay detects physiological changes in Ptc1 and Smo activity states.

Our control experiments confirmed that Ptc1 inhibition is specific to Smo (Fig. S2B), ruling out artifactual effects of Ptc1 on 7TM protein function in general. Although we used the Smo–Go\(_{i}\) fusion in our cell-based experiments for consistency with the in vitro measurements described below, we observed
Our sensor now provides an opportunity to study the kinetics of Ptc1 action by comparing the effects of a direct Smo agonist to Hh-mediated loss of Ptc1 inhibition. We found that acute addition of ShhN to cells coexpressing Ptc1 and Smo activates G proteins on a similar time scale as SAG21k (Fig. 2B), indicating that the Ptc1 affects Smo conformation in a fast, nonrate-limiting manner. From these data, we calculated a $t_{1/2}$ for Smo-mediated G protein coupling (the time required for half-maximal inhibition of cAMP to baseline levels, defined by Smo in the absence of Ptc1) as 3.97 min for ShhN and 2.63 min for SAG21k. As predicted, this is markedly faster than Smo ciliary accumulation ($t_{1/2} \approx 2\text{ h}$) (see ref. 36), a more indirect metric for activity-dependent changes in Smo conformation.

In summary, our rapid cell-based G protein-coupling assay not only faithfully recapitulates the major findings from conventional cell-based transcriptional assays, but also demonstrates that Smo can attain a constitutively active state outside the cilium, likely within the plasma membrane. Thus, even though Ptc1 regulates Smo within the cilium, the underlying mechanism uses factors that are not restricted to this compartment. Furthermore, Ptc1 and Smo change conformation on a time scale significantly faster than is evident from traditional Hh pathway readouts.

**Cholesterol Is Necessary and Sufficient for Constitutive Smo Activation in Vitro.** Our preceding experiments establish that G protein coupling serves as a robust measure of Smo activity that accurately recapitulates its fundamental pharmacological properties, providing snapshots of the active and inactive Smo conformational states that are likely involved in physiological coupling to Gli transcription factors. Unlike conventional Hh pathway functional readouts, however, G protein coupling has the distinct advantage that it can be easily measured in vitro. We capitalized on this property, developing cell-free and reconstituted preparations to study how membrane cholesterol and other factors influence Smo activity.

We began our in vitro studies by constructing and characterizing an optimized assay for in vitro Smo G protein coupling. We adapted a previously described approach (30) using the Smo–G${\alpha}_s$ 

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**Fig. 1.** A live-cell assay shows rapid, cilium-independent Ptc1 regulation of Smo. (A) Schematic diagram of the GloSensor cAMP assay. Smo couples to endogenous (or fused) inhibitory Go proteins, which block forskolin-induced AC activity. Increased Smo activity is therefore reflected as a decrease in luminescence. (B) Live-cell luminescence traces from HEK293 cells transfected with the indicated plasmids. Baseline luminescence was recorded for 10 min at 2-min intervals, followed by forskolin treatment and continued monitoring. For ShhN treatment, ShhN was added 10 min before measurement of baseline and forskolin-induced luminescence; ShhN remained present at 200 nM for the entire experiment. (C) Bar graph shows steady state luminescence for HEK293 cells transfected with GloSensor, Smo, and Ptc1 expression plasmids. Cells were preincubated for 10 min with ShhN or SAG21k as indicated in B.

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**Fig. 2.** Ptc1-mediated regulation of Smo conformation is fast, nonrate-limiting, and occurs independently of Smo ciliary trafficking. (A, Top) Ptc1 blocked the activity of full-length Smo (Smo (FL)) and the cytoplasmic domain (Smo) to similar degrees in the GloSensor assay. (Bottom) A topology diagram of the two Smo expression constructs; the cytoplasmic tail required for transcriptional coupling and ciliary trafficking is indicated in orange. (B) To demonstrate the rapid onset of ShhN or SAG21k effects, cells expressing GloSensor, Smo, and Ptc1 were stimulated with forskolin, and ShhN or SAG21k (vs. a vehicle control) were then added as indicated by the gray bar. (C) Overnight pretreatment with PTX (100 ng/mL) prevents Smo from inhibiting GloSensor luminescence.
fusión, que dramaticamente incrementa la eficiencia de las medidas in vitro comparada con proteínas no fusadas (38). La proteína fusada G actúa como un tartero biosensor que reporta sobre la activación del Smo en la forma de un equilibrio de nucleotídico no hidrolizable GTP (S5-GTPyS) (Fig. 3A). En las células HEK293 transfundidas, Smo-GG̃, muestra una gran actividad constitutiva que es bloqueada por un tratamiento protéico inversor KAAD-cyclopamine y es modulada de forma comparable por el antagonista Smo SAG21k (Fig. 3B), incluso en un modelo de células viables (Fig. 3C y D). Un panel de moléculas de pequeño tamaño de Smo ligand (39) revela una correlación excelente entre los datos de respuesta del Smo G protein coupling y la regulación transcripcional (Fig. S3A). Por lo tanto, con el GloSensor, nuestra metodología de interpolación indica que las conformaciones activas e inactivas son similares. Consistente con este hecho, una molécula Smo-G protein fusion incluyendo el full-length cytotail (SmoFL–GG̃) fue capaz de estimular la transcripción Gli-dependiente (como el C-terminally truncado Smo–GG̃), pero exhibió una actividad basal comparable y una molécula de pequeña escala de molécula modulante como Smo–GG̃ en el G protein-coupling assay (Fig. S3 B y C).

Curiosamente, Smo agonistas y antagonistas mostraron efectos débiles en nuestro sistema in vitro a menos que suplementáramos los buffers con GDP de alta concentración (10–100 μM) GDP (Fig. S4), que incrementa la sensibilidad del G protein a un rango que permite responder eficazmente a cambios conformacionales del Smo. Esta diferencia proporciona una explicación plausible para los efectos adversos de Smo antagonistas y su sinergia con Ptch1 en el GloSensor. Como GDP no puede ser efectivamente alterado en células, las moléculas de la proteína Smo son similares a las de la proteína G protein-coupled receptors (GPCRs) (46). Alternativamente, la función de esterola en Smo y la función de G protein-coupled receptors (GPCRs) (46). Alternativamente, Smo agoine activado por un endógeno ligando, pero también es suficiente para inducir actividad Smo constitutiva, sin embargo, tampoco afecta a Smo activity in cells (21, 22, 40). Nuestros datos indican que Smo sensitivity to steroid depletion in vivo derives from a requirement for cholesterol itself, and not a cholesterol metabolite. As cholesterol is by far the most-abundant sterol in metazoan plasma membranes (cholesterol:phospholipid ratio = 50%) (45), our in vivo results argue that cholesterol will likely play a similar role in determining Smo activity in cells (21, 22, 24, 40).

El rol de esterola en la actividad de Smo es consecuencia de una acción transaccional que promueve la actividad de Smo estimulada por un endógeno ligando, similar a una gran cantidad de receptores de proteína activados por ligando (GPCRs) (46). Alternativamente, Smo alone might suffice to activate Smo. To more directly examine the role of cholesterol in Smo activation, we assayed Smo conformation in a precisely controlled membrane lipid environment. We purified Smo-GG̃ in monodisperse form (Fig. S5) and reconstituted it into lipoproteínas nanodiscs (47, 48) using one of two definidos. When we performed our nanodisc reconstruction, several lines of evidence indicate that cholesterol is not just necessary, but also sufficient to induce constitutive Smo activity, with no requirement for additional proteins and lipids. Cholesterol effects in nanodiscs may derive from a direct interaction between cholesterol and Smo. Several works (23, 25, 27) have reported that cholesterol can interact with the extracellular Smo N-terminal CRD, and recent in vivo labeling studies.
(26) suggested that cholesterol might become covalently attached to the CRD under some circumstances. We found, however, that CRD-deleted Smo also depends on cholesterol for constitutive activity in nanodiscs (Fig. 4B). Furthermore, cholesterol activates Smo over a nearly identical concentration range both in the presence or absence of its CRD (Fig. 4C). We observed similar results with a CRD point mutant (D99A Y134F, hereafter referred to as DAYF) that cannot bind an alkynyl cholesterol derivative in vitro (Fig. S6) (27; see also ref. 26). Finally, by testing a variety of sterols for their ability to rescue Smo GTPyS binding in cyclodextrin-depleted membranes, we noted a distinct structure–activity relationship from that previously described for CRD-sterol interactions (Fig. 5A); 22(S)-OHC fully rescues the effect of sterol depletion in our assay but does not bind the CRD (49), whereas 20(S)-hydrocholesterol [20(S)-OHC], which binds the CRD, is less efficient. Furthermore, the effects of sterols in these experiments are strikingly similar for both wild-type and CRD-deleted Smo (compare Fig. 5A and B), illustrating that the CRD is not required. Taken together, these data indicate that cholesterol can affect Smo activity via its heptahedral bundle. How cholesterol affects the Smo 7TM domain is unclear, but one possibility is that it might engage a specific binding site outside the “cyclopamine pocket” (22, 40).

**Ptch1 Can Regulate Smo Independently of any CRD–Cholesterol Interactions.** Cholesterol exerts at least two distinct effects on Smo activity, one through the CRD and the other through the 7TM domain (as highlighted by our in vitro studies described above), but the key outstanding question is whether Ptch1 inhibits Smo by altering either of these modes of cholesterol interaction. Several recent studies have provided new data in this regard. Luchetti et al. (25) show that an experimentally induced increase in cellular cholesterol, via loading of cholesterol–cyclodextrin complexes into cholesterol-replete fibroblasts, elicits activation of wild-type Smo but not the DAYF mutant (see also ref. 26). Because Smo DAYF also shows reduced activation of Gli transcription in response to loss of Ptch1, it is tempting to speculate that Ptch1 inhibits Smo by somehow operating directly on cholesterol bound to its CRD (Fig. S7A, model 1). Such a model, however, conflicts with the finding that transfected Ptch1 can suppress the activity of a CRD-deleted Smo mutant (22), implying that Ptch1 can regulate Smo independently of the CRD. These seemingly disparate results might be reconciled if CRD binding to cholesterol instead functions to stabilize the constitutively active Smo conformation attained upon loss of Ptch1 inhibition. In this scenario, Ptch1 does not block Smo by altering CRD-cholesterol binding, but rather acts at a site elsewhere on Smo, while CRD–cholesterol interactions serve an auxiliary role by stabilizing an active Smo conformation (Fig. S7A, model 2).
We devised an experiment to distinguish these two models. We combined the DAYF mutation with SmoA1, a 7TM domain mutation that constitutively activates Smo independently of the CRD (15), thereby bypassing the need for any CRD-mediated stabilization of the Smo active state, while still permitting repression by exogenous, transfected Ptch1 (15, 22, 50, 51). If Ptch1 operates directly on a CRD-cholesterol interaction (model 1), Ptch1 transfection should not repress SmoA1–DAYF. In contrast, if the site of Ptch1 action lies outside the CRD, exogenous Ptch1 should block the activity of SmoA1–DAYF (model 2). We found using a Gli transcriptional reporter assay that the constitutive activity of SmoA1–DAYF was dramatically suppressed by Ptch1 transfection (Fig. 6A). This effect closely resembled the action of endogenous Ptch1 on wild-type Smo, as the resulting inhibition was fully reversed by treatment with ShhN (Fig. 6A) in a dose-dependent manner (Fig. 6B). These data indicate that the actions of transfected Ptch1 on Smo in our assays are reflective of true Ptch1 functionality rather than nonspecific consequences of protein overexpression. All of the above results are difficult to reconcile with direct action of Ptch1 via the Smo CRD (model 1), but are readily explained by action of Ptch1 via the Smo 7TM domain, with a critical stabilizing role for the CRD (model 2).

Whereas the experiments presented above involved overexpression of Ptch1 along with a compound SmoA1 mutant, we also observed that endogenous Ptch1 can regulate Smo DAYF even without the activating SmoA1 mutation (Fig. S7B; see also figure 3F in ref. 27); as predicted, the maximal extent of Hh-induced activity is less than its wild-type counterpart because the mutations compromise the CRD’s ability to stabilize active Smo. In contrast, Smo DAYF is completely insensitive to the oxysterol 20(S)-OHC (Fig. S7B), confirming that these mutations disrupt CRD-sterol binding and providing further evidence that Ptch1 effects are separable from CRD–lipid interactions. We also note that whereas wild-type Smo and the DAYF mutant are activated to similar extents by the 7TM agonist SAG (Fig. S7B) (see also refs. 25 and 27), this finding does not rule out model 2 because SAG does not require the CRD to stabilize an active Smo conformation. Finally, Ptch1 was able to repress SmoΔCRD (Fig. 6C) and Smo DAYF (Fig. S7C) in our GloSensor assay, consistent with transcriptional data presented here and in our previous study (23).

We conclude from these experiments that Ptch1 is capable of acting outside of the Smo CRD, likely within the 7TM domain at a site that remains to be mapped. One reason that other studies may not have reached these conclusions is that the Ptch1 responsiveness of CRD-mutated Smo alleles becomes particularly obvious after manipulating Ptch1 levels over a broad range (graphically summarized in Fig. 6D). This is only possible by introducing exogenous Ptch1 via transfection as in the present study (see also refs. 22 and 51), whereas other investigations (23, 25, 27) have sampled a relatively narrow range of Ptch1 activity by relying exclusively on endogenous Ptch1 protein, which is expressed at low levels.

**Ptch1 Regulation of Smo G Protein Coupling Depends on Extracellular Na\(^+\)**. Smo constitutive activity in our GloSensor experiments is dramatically reduced when Ptch1 is cotransfected (Fig. 1). In contrast, we failed to observe any Ptch1-mediated reduction in Smo GDP/GTP\(_{\gamma}\)S binding in membrane fractions (Fig. S8A) or detergent-permeabilized cells (Fig. S8B). This result suggests that Ptch1 activity is not recapitulated in these preparations; and must somehow be lost during the process of cell disruption. The lack of Ptch1 activity following cell breakage might reflect, for example, requirements for intact cellular structure, diffusible cytoplasmic factors, or transmembrane ion gradients in Ptch1 function. In this regard, it is noteworthy that Ptch1 is predicted to function as a transmembrane transporter based on its homology to prokaryotic H\(^+\) gradient-driven RND small-molecule efflux pumps (15). In fact, a recent crystal structure of NPC1 (52), a putative cholesterol transporter and the closest vertebrate Ptch1 relative, reveals that the overall fold and the positions of key amino acids mediating transmembrane H\(^+\) transfer in prokaryotic RNDs (53, 54) are highly conserved (Fig. 7A). Neutralizing the analogous Ptch1 residues (D499N, D500N, E1081Q, hereafter referred to as NNQ) (Fig. 7B) inactivates Ptch1 transmembrane (Fig. 7C) and G protein-coupling assays (Fig. 7D). These findings are consistent with previous reports implying that Ptch1, like its bacterial counterparts, is powered by an ion gradient (15). But while bacterial RNDs rely primarily on a large H\(^+\) gradient between cytoplasm and periplasm (16, 55), no such pH difference exists across the plasma membrane of most metazoan cells. Thus, the energy source for Ptch1 activity is still

**Fig. 6.** Ptch1 can inhibit Smo in the absence of CRD-cholesterol binding. (A) Smo-/- mouse embryonic fibroblasts (MEFs) were transfected with the indicated Smo and Ptch1 expression constructs along with an 8xGli-luciferase reporter, and stimulated with control (black) or ShhN (green) conditioned medium. Relative luciferase units (RLU) are plotted as a fold-increase over the baseline value, defined as reporter activity from the negative control transfection (no Smo). (B) A similar experiment, in which cells transfected with wild-type Smo (red) or SmoA1 D99A Y134F (SmoA1 DAYF) + Ptch1 (blue) were stimulated with increasing concentrations of ShhN conditioned medium. (C) SmoΔCRD is constitutively active and suppressible by Ptch1. Suppression is evident when Ptch1 is coexpressed according to standard conditions (Materials and Methods), and becomes even more obvious when increasing amounts of Ptch1 cDNA are transfected. The standard 10-min ShhN pretreatment fully reverses the effects of Ptch1 coexpression on both wild-type and CRD-deleted Smo, even at the highest amounts of Ptch1 cDNA transfected. The observed suppression of SmoΔCRD is therefore truly dependent on Ptch1 activity and not simply an artifact of protein overexpression in this assay. (D) Simulation of the functional behavior of wild-type and various CRD-mutated forms of Smo, taking into account observations from Gli transcriptional assays in this and several other studies (22, 27, 68). Smo activity is represented on the y axis, while Ptch1 activity (blocked by binding of Hh) is on the x axis. The possible ranges of Ptch1 activity encompassed by endogenous vs. transfected Ptch are indicated below the x axis.
unclear, and any active-transport-based models for Ptc1 function remain largely speculative.

We hypothesized that a different ion might drive Ptc1 enzymatic activity. One of the most fundamental chemiosmotic differences across animal cell membranes is low Na\(^+\)/high K\(^+\) inside and high Na\(^+\)/low K\(^+\) outside the cell (56). We asked whether Ptc1 is inactivated when these gradients are eliminated by bathing cells across animal cell membranes is low Na\(^+\)/high K\(^+\) inside and high Na\(^+\)/low K\(^+\) outside the cell (56). We asked whether Ptc1 is inactivated when these gradients are eliminated by bathing cells

No K\(^+\) gradients likely exerts a multitude of pleiotropic effects on cellular metabolism and physiology, any of which could interrupt the complex, multicomponent ciliary trafficking process (14).

To circumvent this issue, we attempted to assess the effect of manipulating Na\(^+\) and K\(^+\) concentrations on Ptc1 in the rapid, membrane-proximal GloSensor assay. Remarkably, we found that changing the extracellular bath to a low Na\(^+\)/high K\(^+\) solution reversed Ptc1-mediated Smo inhibition, within minutes (Fig. 8A). Conversely, Ptc1 inhibition was briskly restored upon switching from a low Na\(^+\)/high K\(^+\) bath to a physiological high Na\(^+\)/low K\(^+\) bath (Fig. 8B and Fig. S10A). Thus, Ptc1 regulation of Smo requires normal extracellular Na\(^+\) and K\(^+\) concentrations. In similar experiments, we found that replacement of Na\(^+\) with Li\(^+\) in the bath also eliminated Ptc1 inhibitory effects (Fig. S10B). We observed little or no effect of changing the ionic composition of the bath in cells expressing Smo alone (Smo suppression of GloSensor activity remained), in cells expressing GloSensor alone, or in cells expressing the muscarinic acetylcholine receptor, a control GPCR that also affects cAMP levels (Fig. 8C). These control experiments (Fig. 8) also indicate that the GloSensor readout remains robust when transmembrane ion gradients are acutely manipulated, in marked contrast to the Smo ciliary accumulation assay (Fig. S9). A low Na\(^+\)/high K\(^+\) extracellular solution will affect both transmembrane Na\(^+\) and K\(^+\) gradients as well as depolarize the plasma membrane (56) (cytoplasm typically \(-30\) to \(-60\) mV with respect to extracellular space, largely set by the K\(^+\) equilibrium potential). Substituting Na\(^+\) with the organic cation N-methyl-D-glucamine (NMDG\(^+\)), which maintains transmembrane K\(^+\) gradients and perturbs membrane potential only minimally (57), also inactivated Ptc1 (Fig. 8).

![Fig. 7. Ptc1 activity depends on conserved residues that mediate intramembrane ion flux in bacterial RNDs.](image)

![Fig. 8. A role for extracellular Na\(^+\) in Ptc1-Smo regulation.](image)
Gradient to power its transport. Alternatively, extracellular Na+ might somehow act as a cofactor required to maintain Ptch1 or Smo in active or inactive conformations, respectively. Several control experiments argue against the latter possibility. First, GTP/S binding of Smo in cell-free membrane fractions or purified nanodiscs, normally measured in a high Na+/low K+ buffer, is no greater in a high K+/low Na+ buffer (Fig. S10C), and SANT-1 can still block Smo G protein coupling when cells are bathed in high K+/low Na+ (Fig. S10D). Indeed, while ligand binding to some but not all class A GPCRs is modulated by changes in Na+ binding near the GPCR orthosteric site, the critical residues involved in Na+ coordination are not conserved in the Smoothened/Frizzled subfamily (58), consistent with the lack of Na+ effects in the experiments described above. These data demonstrate that depletion of extracellular Na+ is unlikely to activate Smo directly. Finally, Ptch1 can still bind Hh when cells are bathed in high K+/low Na+, arguing that these ion substitutions do not affect Ptch1 folding or stability (Fig. S10E).

The above data are consistent with the idea that Ptch1 activity relies on transmembrane sodium gradients (Fig. 9). Our results, however, do not exclude the possibility that Ptch–Smo regulation requires extracellular Na+ in a manner that is unrelated to transmembrane ion gradients. Definitive proof that Na+ gradients provide the energy source for Ptch1 function awaits demonstration of Ptch1-mediated transmembrane Na+ flux, which will require the development of a direct Ptch1 transport assay. Nevertheless, our findings should provide a useful starting point to investigate Ptch1 using cellular physiology-based approaches.

Discussion

Genetic and cell biological studies clearly support a role for primary cilia in the vertebrate Hh cascade (14), but the precise function of this organelle has remained speculative. We find that Ptch1–Smo regulation can be studied using simple, direct assays that do not depend on cilia. Our measurements show that Ptch1 can switch Smo conformational state within minutes. This time scale is faster than cilia-dependent assays such as the activity-dependent accumulation of Smo in primary cilia (36, 37), as ciliary accumulation not only reflects Smo conformational state but also incorporates downstream, rate-limiting alterations in cilary trafficking following the initial Smo conformational changes.

The cilium is likely the subcellular location where Ptch1 regulates Smo conformation to modulate Gli transcriptional coupling, as all three proteins localize to this compartment and blockade of ciliary biogenesis or trafficking inhibits transcriptional activation. However, our observation that Ptch1 can regulate Smo G protein coupling independently of cilia implies that the underlying mechanism does not require a specialized ciliary environment, and instead must use factors present both inside and outside cilia. Rather than providing an obligate, privileged setting for Ptch1–Smo regulation, the cilium may instead be uniquely required as a “meeting place” to concentrate Ptch1 and Smo with downstream pathway components, thereby permitting efficient coupling to transcriptional effectors. Nevertheless, the ciliary compartment does possess a distinct lipid repertoire (59–61), and cilium-specific lipids might play physiologically relevant roles in fine-tuning the core Ptch1–Smo regulatory step that we have recapitulated in our experiments. Our Smo nanodisc reconstitution system may allow direct testing of this hypothesis in the future. In the long term, it will be important to extend our findings to the endogenous Hh pathway in living cells by developing biochemical and imaging-based tools to directly interrogate Smo conformation in real-time within its native ciliary environment. It may also be worthwhile to extend our studies to other model systems such as the Drosophila Hh pathway, which functions independently of primary cilium, once a reliable short-term readout for Drosophila Smo activity has been developed.

A potential limitation of our approach is that it relies on G protein coupling as a Smo conformational readout, and the contribution of G proteins to Smo regulation under physiological conditions remains a matter of debate. Nevertheless, the G protein-based assays in our study reliably reflect all of Smo’s hallmark functional properties (modulation by established small molecules, inhibition by cholesterol depletion, and sensitivity to Ptch1), arguing that our findings represent a useful, valid framework for future investigations of Smo regulation.

Previously, it was not known whether Ptch1 inhibits Smo by removing an activator or providing an inhibitor, as either scenario is consistent with established genetic relationships. However, our nanodisc reconstitution (Fig. 4) shows that cholesterol is not merely a permissive factor that facilitates the action of another endogenous agonist, but is sufficient in its own right to stimulate Smo activity. This stimulation does not require the CRD, and so cannot be mediated by cholesterol bound to this domain, highlighting the importance of the 7TM region in cholesterol regulation of Smo. Furthermore, Smo shows similar constitutive activity in cell-derived membrane fractions (Fig. 3) and intact cells lacking exogenous Ptch1 (Figs. 1 and 2), even though both of these systems possess an extensive repertoire of lipid species besides cholesterol that could in principle influence Smo activity. Our reconstitution studies thus nominate cholesterol as the most likely substrate for Ptch1 action, if Ptch1 indeed functions by removing a Smo activator. Our in vitro experiments complement prior studies in living systems demonstrating that cholesterol biosynthesis is essential for Smo-mediated signaling (21, 22, 24, 40), and that addition of exogenous cholesterol to cells can promote Smo activity and enhance the ability of Hh to derepress Ptch1 (23, 25). In this regard, a salient feature of in vitro reconstitution is its ability to rigorously establish which components are truly sufficient for a biological process. This overcomes an inherent limitation of previous studies performed within the complex, incompletely defined environment of living cells.
How might Ptch1 act on cholesterol to influence Smo activity? Ptch1 might directly affect an interaction between membrane cholesterol and the Smo 7TM domain (Fig. 9), thereby coopting a near-universal mediator of GPCR stability (46) to allow for robust Hh-mediated regulation of Smo conformation. How Ptch1 could operate to efficiently deplete such a ubiquitous lipid is not obvious, but colocalization of Ptch1 and Smo within a specific membrane domain or a transient Ptch1–Smo interaction might allow Ptch1 to adjust cholesterol concentrations or the inner vs. outer leaflet distribution of cholesterol (62) selectively within the Smo vicinity. Alternatively, Ptch1 might catalyze the accumulation of an as yet unidentified negative regulator that blocks Smo activity by overriding the positive effects of membrane cholesterol (Fig. 9). Future studies using the approaches described here may help to fully define the molecular mechanism of Ptch1–Smo communication, including the identity of any endogenous Ptch1-regulated ligands.

Several recent studies (23, 25–27) demonstrated an intriguing capacity of cholesterol to interact with the Smo CRD, raising the possibility that Ptch1 might regulate Smo activity by changing this extracellular domain’s cholesterol occupancy. However, transcriptional reporter assays, both here (Fig. 6) and in prior studies (22, 24, 27), along with our reconstitution (Fig. 4) and GloSensor (Fig. 6) data, all demonstrate that the Smo CRD is not absolutely required for sensitivity to either cellular cholesterol or Ptch1 action. Instead, we suggest that any effects of Ptch1 on Smo-associated cholesterol proceed via a site within the Smo 7TM domain. The location of this site is unknown, but may be revealed through future mutagenesis, crystallographic studies of active Smo, or computational prediction of lipid binding surfaces (63). Whereas the CRD is not required to achieve Ptch1 regulation of Smo, CRD interaction with cholesterol may help stabilize the active conformation of Smo to an extent that is critical for in vivo pathway function, as suggested by the severe developmental phenotype of a Smo D99N mutant mouse (26). In addition, our data do not exclude the possibility that Ptch1 directly operates on both the CRD and 7TM domains simultaneously, or that the CRD renders Ptch1 action on the 7TM domain more efficient. Nevertheless, our study highlights a direct role for the Smo heptahelical domain as a critical mediator of Ptch1 action. To fully understand how endogenous metabolites regulate Smo, it will be essential to move forward to distinguish between the various sites on which Ptch1 directly operates versus “secondary” sites that are required to stabilize active Smo (which either constitutively bind a cofactor or whose occupancy changes as a downstream consequence of Ptch1 activity on its primary site). By developing direct assays for Ptch activity that do not depend on Smo for a functional readout, it may be possible to resolve this dilemma in the future.

Our study shows that cellular Ptch1 function requires extracellular Na+, leading us to hypothesize that transmembrane Na+ gradients, universally present in metazoans, may supply the energy for Ptch1 action. We cannot at present rule out that the ion substitution effects in our GloSensor assay might reflect a Na+ requirement for Ptch1-Smo regulation that is independent of transmembrane Na+ gradients, in which case Ptch1 activity would rely on some other cellular energy source that remains to be identified. Nevertheless, our data raise the possibility that Ptch1 activity might depend on an ion gradient, implying that the long-hypothesized transporter model for Ptch1 function might be correct. The reliance of Ptch1 on Na+-dependent chemiosmotic gradients, as proposed by Myers et al. (28), might allow Ptch1 to adjust cholesterol concentrations or the inner vs. outer leaflet distribution of cholesterol from lysosomes (18). Given that the lysosomal lumen contains high concentrations of both Na+ and H+ (66), it will be interesting to determine whether the NPC1 protein functions by harnessing the energy from a Na+ gradient.

Seminal advances in many areas of membrane signaling, such as the regulation of GPCRs and ion channels by hormones and neurotransmitters, have relied on rapid, direct, real-time functional readouts in living and cell-free systems. In the past, the question of Ptch1–Smo regulation has not benefited from such approaches. By measuring Smo activity with direct assays in simplified cell-based and in vitro settings, our work now renders the upstream Hh pathway accessible to a variety of experimental treatments that would be impractical with conventional GPCR-dependent readouts. This includes manipulations that, while potentially informative, might kill cells or compromise ciliary trafficking over longer time scales. In addition, our cell-free readouts may allow us to more deeply interrogate Ptch–Smo regulation using in vitro systems where proteins, lipids, and ionic compositions can be rigorously and precisely controlled. We anticipate that such strategies will be generally useful in unraveling the mechanism of Ptch1–Smo communication, potentially revealing fundamental aspects of an enduring mystery in development, cancer, and regeneration.

Materials and Methods

Culture and transfection of Si9 cells, HEK293 fibroblasts, NIH 3T3 fibroblasts, and Smo fibroblasts is described in SI Materials and Methods. G protein coupling in live HEK293 cells expressing Smo or Ptch1 was measured using a cAMP-dependent luciferase (GloSensor 2.2F). Membrane fractions derived from suspension HEK293 cells were analyzed for G protein coupling via 32P-GTP-5 binding and scintillation proximity assay. An N-terminally SBP-tagged Smo-Glo fusion was purified from BadMam-infected suspension HEK293 cells in DDM/CHS detergent, and reconstituted into MSP1D1 nanodiscs, and analyzed for G protein coupling in a similar manner. In experiments studying effects of cholesterol depletion, membrane cholesterol was first depleted from membranes using M/CD, and subsequently restored using various various sterol:M/CD complexes. Di-isoleucine-initiated Shh was purified from bacteria as previously described. Detailed information is provided in SI Materials and Methods.

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