Published in final edited form as:

Dev Cell. 2020 November 09; 55(3): 314–327.e7. doi:10.1016/j.devcel.2020.08.002.

Distinct cation gradients power cholesterol transport at different key points in the Hedgehog signaling pathway

Kostadin Petrov^{1,3}, Bradley M. Wierbowski^{1,3}, Jingjing Liu^{1,2}, Adrian Salic^{1,4,*}

¹Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115

²School of Life Sciences, Peking University, 5 Yiheyuan Road, Beijing, China 100871

³These authors contributed equally

⁴Lead Contact

Summary

Cholesterol plays two critical roles in Hedgehog signaling, a fundamental pathway in animal development and cancer: it covalently modifies the Sonic hedgehog (SHH) ligand, restricting its release from producing cells, and directly activates Smoothened in responding cells. In both contexts, a membrane protein related to bacterial RND transporters regulates cholesterol: Dispatched1 controls release of cholesterylated SHH, and Patched1 antagonizes Smoothened activation by cholesterol. The mechanism and driving force for eukaryotic RND proteins, including Dispatched1 and Patched1, are unknown. Here, we show that Dispatched1 acts enzymatically to catalyze SHH release. Dispatched1 uses the energy of the plasma membrane Na⁺ gradient, thus functioning as an SHH/Na⁺ antiporter. In contrast, Patched1 repression of Smoothened requires the opposing K⁺ gradient. Our results clarify the transporter activity of essential eukaryotic RND proteins, and demonstrate that the two main cation gradients of animal cells differentially power cholesterol transport at two crucial steps in the Hedgehog pathway.

In Brief

Petrov, Wierbowski et al. show that eukaryotic RND proteins Dispatched1 and Patched1, key regulators of the Hedgehog developmental signaling pathway, are transporters powered by distinct ion gradients across the plasma membrane: Dispatched1 uses Na⁺ to catalyze cholesterol-modified Sonic hedgehog secretion, while Patched1 uses K⁺ to antagonize Smoothened activation by cholesterol.

Graphical Abstract

*Correspondence: asalic@hms.harvard.edu.

Author Contributions

K.P., B.M.W. and A.S. conceived the project, and designed and performed the experiments. J.L. conceived and performed WNT3A experiments. K.P. and B.M.W. analyzed data and assembled the figures. K.P., B.M.W. and A.S. wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Hedgehog; cholesterol transport; cation gradient; RND transporter; cell signaling

Introduction

The Hedgehog (Hh) signaling pathway is essential in embryogenesis of most metazoans (Ingham and McMahon, 2001; Lum and Beachy, 2004), while aberrant Hh activity is responsible for several cancers, including basal cell carcinoma and medulloblastoma (Rubin and de Sauvage, 2006). When the Hh pathway is not stimulated, the membrane protein Patched1 (PTCH1) (Nakano et al., 1989) inhibits the GPCR-like protein Smoothened (SMO) (Alcedo et al., 1996; van den Heuvel and Ingham, 1996), thus ensuring that the downstream Hh pathway is idle. Signaling is initiated by a secreted Hedgehog ligand, such as the vertebrate prototype, Sonic hedgehog (SHH) (Chang et al., 1994; Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). SHH binds and inhibits PTCH1, causing SMO to become active. Active SMO then triggers activation of the downstream zinc finger GLI proteins (Humke et al., 2010; Tukachinsky et al., 2010), which turn on transcription of specific target genes.

A unique aspect of Hh signaling is the critical involvement of cholesterol at two distinct steps (Fig.1A). First, in signal-producing cells, cholesterol is covalently attached to SHH (Porter et al., 1996), causing it to strongly associate with membranes; thus cholesterol is crucial for regulating SHH production and distribution. Second, in responding cells, cholesterol activates SMO (Huang et al., 2016; Luchetti et al., 2016) by binding to its extracellular cysteine-rich domain (CRD) (Byrne et al., 2016; Huang et al., 2016; Myers et al., 2013; Nachtergaele et al., 2013; Nedelcu et al., 2013); thus cholesterol is required for Hh signal transduction (Cooper et al., 2003).

Each of the two cholesterol-dependent aspects in Hh signaling involves a protein related to the Resistance–Nodulation–Division (RND) superfamily of transporters (Nikaido, 2018). In signal-producing cells (Fig.1B), the RND-related Dispatched1 (DISP1) (Amanai and Jiang, 2001; Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002; Nakano et al., 2004) is necessary for SHH release in a diffusible form capable of long-distance signaling. DISP1 interacts with the cholesteryl moiety of SHH (Creanga et al., 2012; Tukachinsky et al., 2012), transferring it from the membrane to a secreted SCUBE family protein, such as SCUBE2 (Hollway et al., 2006; Kawakami et al., 2005; Woods and Talbot, 2005). In Hh-responding cells (Fig.4A), PTCH1, itself RND-related, is thought to inhibit SMO by transporting cholesterol. The idea that cholesterol is the PTCH1 substrate is supported by the observation of sterol molecules in recent cryo-EM structures of PTCH1 (Gong et al., 2018; Qi et al., 2018a; Qi et al., 2018b; Qian et al., 2019; Zhang et al., 2018), although the manner and direction of the presumed cholesterol movement are unclear.

The RND superfamily includes both prokaryotic and eukaryotic members. Prokaryotic RNDs function as proton (H^+)-driven antiporters, in which expulsion of small molecule substrates from the periplasm of Gram-negatives to the extracellular space is coupled with inward H^+ flow, from the periplasm into the cytoplasm (Aires and Nikaido, 2005; Delmar et al., 2015; Seeger et al., 2006). Eukaryotic RND-related proteins include Niemann-Pick Disease Protein C1 (NPC1) (Carstea et al., 1997) and NPC1L1 (Davis et al., 2004), which play fundamental roles in cholesterol metabolism, and DISP1 and PTCH1, which are essential for embryonic development. In contrast to bacterial RNDs, however, eukaryotic RND-related proteins remain poorly understood mechanistically. Catalytic substrate transport has not been directly demonstrated for any eukaryotic RND, and, furthermore, it is unknown if and how eukaryotic RNDs are powered by ionic gradients. Two residues required for H^+ conductance in bacterial RNDs are conserved and required for DISP1 (Ma et al., 2002) and PTCH1 (Taipale et al., 2002) function; in the case of PTCH1, mutations in these residues cause Gorlin Syndrome, a genetic predisposition to cancers caused by hyperactive Hh signaling (Taipale et al., 2002). However, unlike bacteria, most eukaryotic cells do not maintain an H^+ gradient across the plasma membrane, suggesting that eukaryotic RNDs, including DISP1 and PTCH1, perhaps rely on different ionic gradients. A recent report suggested that PTCH1 activity requires Na^+ gradient (Myers et al., 2017), but this study was not performed in Hh-responsive cells and did not use a physiological Hh readout, leaving unresolved the issue of how PTCH1 is powered in cells undergoing normal Hh signaling.

Here, we address two major open questions concerning eukaryotic RND-related proteins: do they catalyze substrate transport, and what is their energy source? We start by developing a fast and quantitative assay for DISP1 activity, which we use to demonstrate that DISP1 acts catalytically to transport cholesterylated SHH during its release from cells. We find that the plasma membrane Na^+ gradient is absolutely required for DISP1 activity, while the K^+ gradient is dispensable; furthermore, increasing the steepness of the Na^+ gradient potentiates DISP1 activity. Since cholesterylated SHH is released to the extracellular space while Na^+ enters the cell, this indicates that DISP1 functions as a Na^+ -powered antiporter. In contrast, in the case of PTCH1, we find that its inhibitory effect on SMO requires the K^+ gradient, but not the Na^+ gradient; this suggests that PTCH1 operates as a K^+ -powered cholesterol

transporter. Both for DISP1 and PTCH1, mutagenesis and functional assays support the role of a cation different from H^+ . Our results demonstrate catalytic transporter activity for a crucial eukaryotic RND, and show that the two critical steps in the Hh pathway involving cholesterol transport are powered by two distinct monovalent cationic gradients across the plasma membrane.

Results

A quantitative cellular system for DISP1-dependent SHH release

In spite of the critical importance of eukaryotic RND-related proteins, catalytic transporter function has not been directly demonstrated for any of them. To tackle this problem, we focused on developing a biochemical system for measuring DISP1 activity. The choice of DISP1 was based on the following rationales: (1) DISP1 functions at the plasma membrane, thus is immediately accessible to factors added exogenously; (2) the movement of the cholesterylated SHH substrate is well-defined, from the outer membrane leaflet to the extracellular SCUBE2 acceptor; and (3) uniquely among eukaryotic RNDs, DISP1 acts on a cholesterol substrate attached to a protein, which should greatly simplify quantification (Fig.1B).

SHH release by SCUBE2 has been observed in cultured HEK293T cells (Creanga et al., 2012; Tukachinsky et al., 2012), but it is unclear if this process requires endogenous DISP1. We thus generated *DISP1* knockout (*DISP1*^{KO}) HEK293T cells (Fig.S1A), and measured SHH release. As shown in Fig.1C, SCUBE2 did not release SHH from *DISP1*^{KO} cells, in contrast to the robust release observed in parental wild-type cells. Importantly, SCUBE2-dependent SHH release was fully rescued by transfecting *DISP1*^{KO} cells with a construct expressing wild-type DISP1, but not the inactive DISP1-NNN mutant (Ma et al., 2002) (Fig.1C), indicating the specificity of the SHH release defect. Thus SHH release from HEK293T cells recapitulates the strict requirement for DISP1 and SCUBE family proteins observed in *in vivo* studies of the Hh pathway.

In the experiments above, SHH was collected after several hours and then assayed by immunoblotting. To develop a faster and more sensitive system capable of measuring release kinetics, we tagged SHH with the highly active, ATP-independent NanoLuc luciferase (NL) (Hall et al., 2012), as previously done using *Renilla* luciferase (Ma et al., 2002). The NL tag was introduced internally (Fig.S1B, C), to preserve SHH modification with palmitate at the N-terminus (Chamoun et al., 2001; Pepinsky et al., 1998) and cholesterol at the C-terminus (Porter et al., 1996). A total of nine SHH-NL fusions were generated, with different NL insertion sites into SHH (Fig.S1B, C) (Ma et al., 2002), and were screened for SCUBE2-dependent release from cells. As shown in Fig.S1D, release of three of the SHH-NL fusions was stimulated by SCUBE2 co-expression. The two best-behaved fusions, SHH-NL5 and SHH-NL7, were then tested using purified recombinant SCUBE2 (Fig.S1E). As shown in Fig.S1F, SHH-NL5 and SHH-NL7 were released in a SCUBE2-dependent manner, and could be detected in the culture media by luminescence after much shorter times than SHH detection by immunoblot (Fig.1C). Subsequent experiments were performed using SHH-NL5 (Fig.1D). Significantly, SHH-NL5 was released from cells at similar levels and with the same kinetics as untagged SHH (Fig.1E; Fig.S1G–I), and luminescence and immunoblot

measurements of SHH-NL5 were strongly correlated (Fig.S1J). Importantly, SHH-NL5 release from cells requires both DISP1 and SCUBE2 (Fig.1F), indicating its suitability for studying physiological SHH release.

DISP1 catalyzes SHH release from cells

To determine if DISP1 functions as a transporter, we first characterized the kinetics of SHH-NL5 release from cells. To avoid the potentially confounding effect of continued SHH-NL5 production, protein synthesis was blocked with cycloheximide (Fig.2A). Adding a fixed concentration of purified SCUBE2 caused an increase in SHH-NL5 release that was linear with time (Fig.2B, C). Although release by SCUBE2 could be robustly measured after less than 1 minute, strictly linear release continued for the entire 30-minute duration of the experiment (Fig.2B). Furthermore, the 30-minute incubation with SCUBE2 had a negligible effect on cellular SHH-NL5 levels (Fig.S2A, B), indicating that the SHH-NL5 substrate was saturating under these experimental conditions. Similar DISP1- and SCUBE2-dependent initial release rates were observed for SHH(C24A)-NL5, which is cholesterylated but not palmitoylated, consistent with the idea that the SHH cholesterol moiety serves as substrate for DISP1 (Fig.S2C). Together, these results indicate that our system can be used to precisely measure initial rates of DISP1- and SCUBE2-dependent SHH release (Fig.2C).

Upon titration of purified SCUBE2 added to SHH-NL5-expressing cells, we observed a saturable increase in the initial rate of SHH-NL5 release (Fig.2D, E). Importantly, though the rate of release saturated, SHH-NL5 release did not, in accord with our finding that cellular SHH-NL5 levels were not limiting (Fig.S2A, B). This kinetic behavior is not consistent with a simple SHH-SCUBE2 binding equilibrium, where SHH release rate would be a linear function of SCUBE2; it is consistent, however, with an enzymatic process, whereby formation of the SHH-SCUBE2 complex is catalyzed by a saturable (i.e. rate-limiting) enzyme. Indeed, the rate of SHH-NL5 release is fit well by a simple Michaelis-Menten equation ($R^2 = 0.9954$), with the K_M in the low micromolar range of SCUBE2 ($K_m \sim 3 \mu M$) (Fig.2E; see STAR Methods).

We next asked if DISP1 is the enzyme catalyzing SHH release. To this end, we compared the rate of SCUBE2-dependent SHH-NL5 release from cells expressing different levels of DISP1 (Fig.2F-K; Fig.S2H-K). DISP1 was titrated either by expressing additional DISP1 on top of endogenous DISP1 (Fig.2F-H; Fig.S2D, E), by isolating low and high DISP1-expressing cells by fluorescence-activated sorting (Fig.2I-K; Fig.S2F, G), or by expressing DISP1 by transient transfection of DISP1^{KO} cells (Fig.S2H-K). In all cases, the rate of SHH-NL5 release was potentiated by increasing DISP1 levels, and was saturable by SCUBE2 (Fig.2F, I; Fig.S2H). Crucially, increasing DISP1 levels increased the maximal rate of SHH-NL5 release (V_{max}) (Fig.2G, J; Fig.S2I), but not the K_M , which remained 2–3 μM (Fig.2H, K; Fig.S2J); this result strongly implies that DISP1 catalyzes SHH release (see STAR Methods). SHH-NL5 release by DISP1 and SCUBE2 is in stark contrast to SHH-NL5 release by methyl- β -cyclodextrin (M β CD), a sterol-binding cyclic oligosaccharide that efficiently solubilizes cholesterylated SHH from membranes (Tukachinsky et al., 2012). SHH-NL5 release by M β CD was DISP1-independent (Fig.S2L-N), and the rate was a linear function of M β CD concentration, as expected for an uncatalyzed process. Furthermore,

SHH-NL5 release by M β CD was more than an order of magnitude slower, pointing to the specificity of the DISP1–SCUBE2 system for SHH transport.

Finally, if DISP1 acts as an enzyme to catalyze SCUBE2-dependent SHH release, we would expect the formation of a DISP1–SHH–SCUBE2 ternary complex, which predicts release rate saturation at high concentrations of SCUBE2 and SHH-NL5 (see STAR Methods). To test this, we titrated SHH-NL5 expression by transient transfection in cells with endogenous DISP1 (Fig.S2O), and measured the rate of SHH-NL5 release at low (1 μ M) and high (10 μ M) SCUBE2 (Fig.2L). Importantly, at high SCUBE2 concentrations the rate saturated as a function of SHH-NL5. These data are further consistent with DISP1 enzymatic activity and suggest the formation of a ternary DISP1–SHH–SCUBE2 complex. Together, these results indicate that DISP1 catalyzes release of SHH from cells.

The transmembrane Na⁺ gradient is necessary for DISP1 activity

Given the homology between DISP1 and bacterial RND transporters, we asked whether SHH transport by DISP1 is powered by a cation gradient at the plasma membrane. Na⁺ and K⁺ are the two monovalent cations with appreciable concentration gradients across the plasma membrane of metazoan cells; at rest, Na⁺ concentration is high outside the cell and low inside, while K⁺ concentration shows the opposite distribution (Fig.3A). We first measured SHH-NL5 release in cells treated with gramicidin, a pore-forming antibiotic specific for monovalent cations, which abolishes both Na⁺ and K⁺ gradients. As shown in Fig.3B, the SHH release rate was greatly reduced by gramicidin; importantly, gramicidin did not affect cell viability (Fig.S3A) over the course of the experiment. A similar result was observed when cells were incubated in isotonic media in which Na⁺ was replaced with K⁺ (Table S1), a treatment that also diminishes both Na⁺ and K⁺ gradients (Fig.3B). Importantly, the H⁺-specific ionophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP), which equilibrates H⁺ gradients across membranes, had a minimal effect on SHH release (Fig.S3B), thus excluding H⁺ as a potential driver of DISP1 activity. Together, these results suggest that a monovalent, non-H⁺ cation gradient is necessary for SHH release, although they do not identify which ion is involved.

To test the possible role of the Na⁺ gradient, we measured SHH-NL5 release from cells incubated in defined low-Na⁺ media, adjusted to isotonicity with the chloride salts of the cations K⁺, Cs⁺, choline⁺, or N-methyl-D-glucamine (NMDG⁺) (Table S1). In all cases, we observed a dose-dependent decrease in the release rate with decreasing extracellular Na⁺ (Fig.3C; Fig S3C, D). This effect cannot be attributed to decreases in the membrane potential because Cs⁺, choline⁺, and NMDG⁺ ions are not membrane-permeant at short timescales, and thus do not depolarize the cells, as high extracellular K⁺ does (Fig.S3E). Importantly, the observed effect was not caused by cellular toxicity, as shown by measurements of cellular ATP levels (Fig.S3F), and of NAD(P)H levels (Fig.S3G). Furthermore, inhibition of Na⁺-dependent SHH-NL5 release was rapidly reversible: release rate was greatly reduced upon replacement of extracellular Na⁺ with choline⁺, and then quickly recovered (<1min) when cells were returned to media with high extracellular Na⁺ (Fig.3D). Finally, DISP1-independent SHH-NL5 release by M β CD occurred normally in low-Na⁺ media (Fig.S3H), indicating that the SHH-NL5 substrate remains accessible to

extracellular factors, on the cell surface. Together, these results indicate that the Na^+ gradient is required for DISP1-dependent SHH release.

The Na^+ gradient drives SHH release from cells

The acute response of SHH release to transient perturbations of the Na^+ gradient (Fig.3D) suggests that perhaps Na^+ directly drives the function of DISP1. One implication of this hypothesis is that the rate of SHH release should be potentiated by a steeper than normal Na^+ gradient across the plasma membrane. To test this prediction, we measured V_{max} for SHH release when extracellular Na^+ concentration was increased above normal. Since the Na^+ concentrations that we tested cause the media to be slightly hypertonic (370 mOsm), all media used in this experiment were adjusted to the same osmolarity, using either choline⁺ or Cs⁺ cations instead of Na^+ . V_{max} was measured by adding saturating levels of purified SCUBE2 (15 μM , corresponding to ~5 times the K_M value). Strikingly, increasing the strength of the Na^+ gradient accelerated SHH-NL5 release, with the rate displaying a linear ($R^2 > 0.999$) dependence on the Na^+ electrochemical driving force (EDF_{Na^+}) (Fig.3E). Significantly, media in which Na^+ was replaced with choline⁺ do not exhibit changes in membrane potential (Fig.S3I), thus excluding membrane potential change as cause of the accelerated SHH release. We were unable to detect DISP1- and SCUBE2-dependent uptake of radioactive $^{22}\text{Na}^+$ into cells (Lee, 1984a, b); perhaps the DISP1 turnover number is too low relative to background ion fluxes across the plasma membrane.

We performed several experiments to test the specificity of Na^+ perturbations on DISP1- and SCUBE2-dependent SHH-NL5 release. First, we assayed the effect of Na^+ gradient change on residual SHH-NL5 release from DISP1 deficient (DISP1^{KO}) cells (Fig.3F). Second, we assayed the effect of Na^+ gradient change on SHH-NL5 release by M β CD, which is SCUBE2-independent (Fig.3G). Third, we assayed the effect of Na^+ gradient change on the release of NL-tagged WNT3A, a lipidated Wnt family ligand unrelated to SHH (Fig.3H; Fig.S3J–L). Finally, we assayed the effect of K^+ gradient change on DISP1- and SCUBE2-dependent SHH-NL5 release, using hypertonic media in which Na^+ was maintained at physiological levels, but extracellular K^+ was varied (Fig.3I). In none of these experiments did we observe changes in the release rate of either SHH or WNT3A. Together, these results indicate that the Na^+ gradient specifically drives DISP1- and SCUBE2-dependent release of SHH, consistent with DISP1 being powered by Na^+ influx.

The transmembrane Na^+ gradient is not required for PTCH1 activity

We next asked if and how PTCH1, the other RND-related protein involved in Hh signaling, might also act as a cation-driven transporter, a longstanding hypothesis in the field (Ingham et al., 2000; Taipale et al., 2002). In the absence of SHH, PTCH1 localizes to primary cilia (Rohatgi et al., 2007) and prevents accumulation of SMO in cilia. Upon binding SHH, PTCH1 is inhibited and removed from cilia. This results in SMO activation and accumulation in cilia (Corbit et al., 2005), leading to ciliary recruitment and activation of GLI-family transcription factors (Humke et al., 2010; Tukachinsky et al., 2010), which ultimately turn on transcription of Hh target genes, such as *Gli1* (Fig.4A). Since SMO is the most proximal downstream target of PTCH1, and SMO accumulation in cilia is the fastest readout for Hh pathway activation, we used it to measure PTCH1 activity and its sensitivity

to cation gradient perturbations. In some instances, we used additional readouts of SMO and Hh pathway activation, namely ciliary accumulation of GLI proteins and transcription of the Hh target gene, *Gli1*.

SMO and downstream Hh signaling were not activated when the Na^+ gradient was abolished by incubating cells in media in which Na^+ was replaced with choline $^+$ (Fig.4B–E), or NMDG $^+$ (Fig.4E); this shows that, in the absence of the Na^+ gradient, SMO is efficiently repressed, thus PTCH1 is active. Importantly, even in the absence of the Na^+ gradient, the cells retained their ability to robustly activate Hh signaling in response to the small-molecule SMO agonist, SAG (Chen et al., 2002) (Fig.4B–E). Thus, in contrast to DISP1, the Na^+ gradient is not required for PTCH1 function.

The transmembrane K^+ gradient is necessary for PTCH1 activity

Dramatically, replacing extracellular Na^+ with K^+ or Rb^+ caused Hh pathway activation in NIH-3T3 cells, as indicated by ciliary accumulation of SMO (Fig.5A, B; Fig.S4A) and GLI proteins (Fig.5C; Fig.S4B), and stimulation of *Gli1* transcription (Fig.5D). In all cases, the small-molecule SMO antagonist, SANT-1 (Chen et al., 2002), reversed these effects (Fig.5A, C, D; Fig.S4B), indicating that Hh pathway activation was SMO-dependent. SMO activation by high extracellular K^+ was also observed in mouse embryonic fibroblasts (MEFs) (Fig.S4C). Together, these results suggest that the K^+ gradient might be required for PTCH1 function.

We also tested whether reducing the K^+ gradient inhibits PTCH1 reversibly. Hh signaling was activated with SHH, which caused accumulation of SMO in cilia (Fig.5E). SHH was then washed out, and we measured the decay of ciliary SMO, a process dependent on PTCH1 activity. As shown in Fig.5E, ciliary SMO was removed from cilia in media of normal ionic composition, but not in high K^+ media. Importantly, PTCH1 activity was recovered when cells were switched from high to low K^+ , indicating that the inhibitory effect is reversible.

The treatments above alter not only the K^+ gradient, but have other effects on cells, including possible perturbations of ciliary trafficking, energy metabolism, Ca^{2+} levels, and resting membrane potential (E_m). To exclude the possibility that one of these perturbations might have caused SMO activation, we performed a number of control experiments. First, we assayed the effect of high extracellular K^+ or Rb^+ on ciliary trafficking, by measuring PTCH1 removal from cilia upon SHH binding, as well as by assaying the constitutive ciliary localization of serotonin receptor 6 (HTR6). High K^+ or Rb^+ allowed normal SHH-induced PTCH1 exit from cilia (Fig.5F; Fig.S4D), while high K^+ did not change ciliary HTR6 levels (Fig.S4E). These results indicate that high extracellular K^+ or Rb^+ do not generally perturb ciliary trafficking.

Second, we measured cellular ATP levels in cells incubated with high extracellular K^+ or Rb^+ . We found that high extracellular K^+ or Rb^+ reduced cellular ATP by 40–50% compared to low extracellular K^+ (Fig.S3F). We speculate that reduction in cellular ATP may be responsible for the blunted pathway activation that we observe with these treatments; however, it is not sufficient to explain the observed pathway activation.

Third, we investigated whether divalent cations, Ca^{2+} and Mg^{2+} , affect SMO ciliary dynamics, and whether they are required for SMO activation in the presence of high extracellular K^+ or Rb^+ . By incubating cells in media lacking extracellular Ca^{2+} and Mg^{2+} , we observed that extracellular pools of Ca^{2+} and Mg^{2+} are dispensable for SMO inhibition by PTCH1, for SMO activation in response to SHH, as well as for SMO activation by high levels of extracellular K^+ and Rb^+ (Fig.S4F). These data suggest that any putative Ca^{2+} and Mg^{2+} fluxes are not necessary for SMO inhibition by PTCH1.

Fourth, we asked if membrane depolarization caused by high extracellular K^+ or Rb^+ (Fig.S3E), rather than a decrease in K^+ gradient, is the cause of SMO activation. Treating cells with the sodium channel agonist veratridine, which depolarizes cells without collapsing the K^+ gradient (Fig.S4G), led only to a very slight increase in ciliary SMO (Fig.S4H); thus, cellular depolarization cannot account for SMO activation by high K^+ and Rb^+ .

Finally, we discovered that cesium ions (Cs^+) cause dose-dependent accumulation of SMO in cilia (Fig.5B; Fig.S4A, I), which was reversed by SANT-1 (Fig.S4I). Cs^+ was also more potent than K^+ or Rb^+ in activating SMO (Fig.5B; Fig.S4I). Cs^+ did not perturb PTCH1 ciliary trafficking (Fig.5F; Fig.S4D), cellular ATP levels (Fig.S3F), and did not depolarize cells (Fig.S3E), consistent with a specific inhibitory effect on PTCH1. Interestingly, Cs^+ does not collapse the K^+ gradient, suggesting a different explanation for its effect. We note that the potency of PTCH1 inhibition increases as function of van der Waals radius for K^+ , Rb^+ and Cs^+ (Fig.S4J); we speculate that Cs^+ , and to a lesser extent Rb^+ , might inhibit PTCH1 by occluding a putative cation-conducting pore in PTCH1.

A possible mechanism for cation-dependent DISP1 and PTCH1 function

We used structure-guided mutagenesis and functional assays to probe the role of cations in DISP1 and PTCH1 function. Homology between DISP1, PTCH1, and H^+ -powered bacterial RNDs is highest within their transmembrane (TM) domains. In particular, there is striking conservation of three sets of polar residues within helices TM4, TM10 and TM11 (Fig.6A). In AcrB (Murakami et al., 2006; Seeger et al., 2006), these residues form an alternating hydrogen-bonding network (Fig.6B). In the absence of protons, the two neighboring aspartate residues in TM4 (D407 and D408) are ionized and coordinate the ϵ -amino group of the lysine residue in TM10 (K940). In the presence of protons, the glutamic acid residues in TM4 are titrated, and the ϵ -amino group of the lysine in TM10 now interacts with the serine and threonine residues in the neighboring TM11 (T978 and S979). By altering the hydrogen-bonding network of these TM-resident residues, AcrB couples proton binding to conformational changes that enable substrate pumping (Pos, 2009). Consistent with this mechanism, the three sets of polar residues are necessary for AcrB function (Su et al., 2006; Takatsuka and Nikaido, 2006).

While DISP1 and PTCH1 both contain the TM4-resident aspartate found in bacterial RNDs, they lack the opposing, TM10-resident lysine; instead this residue is replaced with an acidic one, either aspartate in DISP1 or glutamate in PTCH1 (Fig.6A). We hypothesize that the triad of acidic residues in TM4 and TM10 of DISP1 and PTCH1 forms a cation-binding pocket, such that, in the cation's presence, a salt bridge forms between TM4 and TM10, analogous to the link between TM4 and TM10 in unprotonated AcrB (Fig.6C). Upon cation

dissociation, the negative charge of these residues would lead to electrostatic repulsion and dissolution of the salt bridge, and formation of a hydrogen bond between the first TM4 aspartate of DISP1 and PTCH1 and the TM11 serine/threonine cluster (Fig.6C). At least for PTCH1, evidence of such an alternating hydrogen-bonding network can be found in recent cryo-EM structures (Fig.S5).

The prediction of this model is that this set of polar residues would be necessary for DISP1 and PTCH1 function. This was shown for the TM4 aspartate residues in both DISP1 (Ma et al., 2002) and PTCH1 (Taipale et al., 2002). Additionally, we find that the TM11 serine/threonine cluster is absolutely required for DISP1 (Fig.6D; Fig.S6A, B) and PTCH1 (Fig.6E; Fig.S6C) function. In accordance with the proposed mechanism (Fig.6C), introduction of a lysine residue (i.e. non-dissociating cation) within the TM4–TM10–TM11 network drastically inhibits both DISP1 (Fig.6D; Fig.S6A, B) and PTCH1 (Fig.6E; Fig.S6C). Finally, we find that low pH inhibits, while high pH potentiates DISP1 activity (Fig.6F), an effect that can be explained by protonation of TM-resident acidic residues inhibiting cation coordination. The observed pH dependence is consistent with the calculated pKa values (Kilambi and Gray, 2012) of the three TM-resident acidic residues in DISP1 (3.8, 3.5, and 4.1, for D571, D572, and D1049 respectively). A similar effect has been observed for the Na⁺-powered human choline symporter (hCHT) (Iwamoto et al., 2006). Together, these data support DISP1 and PTCH1 as transporters powered by non-H⁺ cations, and point to a possible mechanism for how cation-induced conformational changes in the TM domain are translated into substrate pumping.

Discussion

Among signaling pathways that orchestrate animal embryogenesis, the Hh pathway stands out for the critical role played by cholesterol, at two different steps. First, cholesterol is covalently attached to SHH, a unique modification catalyzed by the C-terminal domain of the SHH precursor (Porter et al., 1996). Second, the GPCR-like protein SMO, which conveys signals to the cytoplasm, is directly activated by cholesterol (Huang et al., 2016; Luchetti et al., 2016). In both instances, the pathway employs RND-related proteins: DISP1 interacts with cholesterylated SHH and hands it off to an extracellular SCUBE family protein (Creanga et al., 2012; Tukachinsky et al., 2012), resulting in SHH morphogen release, while PTCH1 interacts with cholesterol and antagonizes SMO, thus repressing Hh signal transduction. Two important open questions are whether eukaryotic RND-like proteins, such as DISP1 and PTCH1, function as transporters, and what is the energy source that powers them. Prokaryotic RNDs use the H⁺ gradient across the plasma membrane (Fig.7A), which does not exist in most eukaryotic cells. Here, we directly demonstrate transporter activity for a eukaryotic RND-like protein, DISP1. We then show that DISP1 is powered by the Na⁺ gradient at the plasma membrane, while the K⁺ gradient is dispensable (Fig.7B). Interestingly, we find that PTCH1 displays the opposite behavior: the K⁺ gradient is necessary for its activity in Hh signaling, but the Na⁺ gradient is dispensable (Fig.7C, D). Thus Na⁺ and K⁺, the two monovalent cations that form substantial gradients across the plasma membrane of animal cells, are used to power DISP1 and PTCH1, respectively. In spite of diverged cation specificity, however, the mechanism by which cation binding is

translated into substrate pumping appears conserved between DISP1, PTCH1 and prokaryotic RNDs, as indicated by our functional studies.

Past investigations of DISP1 (Creanga et al., 2012; Tukachinsky et al., 2012) lacked the sensitivity and temporal resolution needed to address the role of ion gradients. We therefore developed a fast and sensitive NanoLuc luciferase assay, inspired by an earlier *Renilla* luciferase assay (Ma et al., 2002), which allowed us to directly study the kinetics of SHH substrate release by DISP1 and purified SCUBE2, in media of defined ionic composition. Our measurements indicate that DISP1 is a transporter quantitatively described by Michaelis–Menten kinetics, with a catalytic rate constant (k_{cat}) that is a function of the transmembrane Na^+ gradient. Strikingly, a steeper Na^+ gradient increases k_{cat} and accelerates SHH release, indicating that the Na^+ gradient directly controls DISP1.

Why would DISP1 need energy to catalyze SHH release, given that SHH is likely more concentrated on the membrane than in the extracellular space? We speculate that the cholesteryl anchor of SHH is strongly attached to membrane lipids, resulting in high activation energy for direct SHH transfer to SCUBE2. To catalyze SHH transfer, DISP1 would not only need to provide a pathway for the cholesteryl anchor to SCUBE2, but also to overcome the binding energy between anchor and membrane. Importantly, energy consumption would ensure that SHH transfer by DISP1 is unidirectional. Similar considerations apply for energy utilization during cholesterol transport by PTCH1 (see below).

The present kinetic data and previous crosslinking experiments using SHH modified with photoreactive cholesterol analogues (Tukachinsky et al., 2012), are consistent with the formation of a DISP1–SHH–SCUBE2 complex. SHH transfer from DISP1 to SCUBE2 resembles, in reverse, the transfer of cholesterol between the soluble NPC2 protein and NPC1 (Infante et al., 2008), for which a stable NPC1–NPC2 interaction has been observed (Deffieu and Pfeffer, 2011). We have so far been unable to detect DISP1–SCUBE2 binding, even in presence of SHH, possibly because such an interaction is transient. Further studies will be necessary to dissect SHH transfer from DISP1 to SCUBE2, and to capture and structurally characterize a possible ternary complex.

Unlike DISP1, the mode and direction of cholesterol transport by PTCH1 are unclear (see below). Thus, instead of directly assaying substrate transport by PTCH1, we measured PTCH1 activity using endogenous downstream readouts of the Hh pathway, primarily the activity state of SMO. We find that PTCH1 represses SMO efficiently and keeps the Hh pathway off even when the Na^+ gradient is abolished, in contrast to DISP1. Strikingly, abolishing the K^+ gradient leads to PTCH1 inhibition and Hh pathway activation, as assayed by SMO and GLI recruitment to primary cilia, and activation of target gene transcription. Importantly, other effects can be excluded as the cause of Hh pathway activation, including membrane depolarization, energy metabolism, or effects on ciliary trafficking. These results indicate that the K^+ gradient powers PTCH1 activity.

Our observation that the Na^+ gradient is dispensable for SMO repression by PTCH1 contrasts with a recent report claiming that PTCH1 activity requires the Na^+ gradient (Myers

et al., 2017). Although the reason for this discrepancy is not clear, it might be due to one of several differences in experimental design. We used MEFs that display robust cilia-dependent Hh signaling, while the other study used HEK293T cells, which lack endogenous Hh signaling. We assayed endogenous SMO, while the other study assayed truncated SMO fused to Gai, a construct of unclear physiological relevance in Hh signaling. Finally, we used physiological readouts of SMO activation, while the other study measured the reduction of forskolin-enhanced cAMP levels.

Our results clarify the relationship between the direction of substrate transport and ion flow in eukaryotic RND-related proteins. In the case of DISP1, cholesterylated SHH is transported from the membrane to the extracellular space, while Na^+ ions flow along their concentration gradient, from the extracellular space inward. Thus DISP1 is a Na^+ /SHH antiporter, similar to bacterial RNDs, which are H^+ /substrate antiporters, pumping substrates out of the cell while H^+ ions flow inward.

In the case of PTCH1, the pathway of cholesterol transport remains unresolved, a question with important implications for the mechanism of SMO repression. One suggestion is that PTCH1 moves cholesterol from the inner to the outer leaflet of the plasma membrane (Zhang et al., 2018); however, the imaging method for measuring cholesterol used in this study has been questioned, due to its strong dependence on the phospholipid composition of the membrane (Courtney et al., 2018). If PTCH1 is an antiporter, the fact that it is powered by the K^+ gradient implies that it must transport cholesterol toward the cell, while K^+ ions flow outward; this is similar to how cholesterol movement occurs in the NPC2–NPC1 system, from the lysosomal lumen into the limiting membrane. For a PTCH1 K^+ /cholesterol antiporter, we envision two possibilities: PTCH1 moves cholesterol from the outer to the inner leaflet of the membrane (Fig.7C), or PTCH1 accepts cholesterol directly from SMO, perhaps from the CRD, and transports it to the outer leaflet of the membrane (Fig.7D). The former model implies that PTCH1 prevents SMO activation by cholesterol, for example by lowering cholesterol concentration in the ciliary membrane. In contrast, the latter model implies that PTCH1 catalyzes deactivation of cholesterol-bound SMO. We note that cholesterol transport postulated by this latter model is consistent with recent cryo-EM studies (Gong et al., 2018; Qian et al., 2019), in which sterol densities are observed both in the extracellular domain and in an outer leaflet-proximal groove of PTCH1, suggesting a conduit from SMO to the membrane. An attractive aspect of this model is that it explains the specificity of PTCH1 for SMO and the Hh pathway, in contrast to a model in which PTCH1 transports cholesterol independent of SMO. Another implication of this model is that SMO can bind cholesterol early in the secretory pathway, and PTCH1 would remove it only when SMO reaches the cilium. Importantly, even though cholesterol-bound SMO might adopt an active conformation outside the cilium, it would not trigger Hh signaling, as that requires the presence of cilia-localized pathway components. Functional experiments will be required to elucidate this important aspect of the PTCH1–SMO interaction.

A useful estimate is that of the free energy that transmembrane Na^+ and K^+ gradients can provide for cholesterol transport by DISP1 and PTCH1, respectively. The power of an ionic gradient is expressed as Gibbs free energy, which can be calculated according to equation (1):

$$\Delta G_{\text{ion}} = zF(\text{EDF}_{\text{ion}}) \quad (1)$$

where z is the ion charge, F is the Faraday constant, and EDF_{ion} is the electrochemical driving force. In a typical fibroblast ($E_m = -54\text{mV}$, $[\text{Na}^+]_{\text{out}} = 150\text{mM}$ and $[\text{Na}^+]_{\text{in}} = 15\text{mM}$) the Na^+ gradient has an $\text{EDF}_{\text{Na}^+} = -115\text{mV}$, which translates into $\sim 11\text{kJ/mol}$ of free energy available for Na^+ -powered transport by DISP1. In the case of PTCH1, we must consider that it functions at the ciliary membrane (Rohatgi et al., 2007), which has electrophysiological properties slightly different from the plasma membrane (Delling et al., 2013); specifically, at the primary cilium, $E_m = -20\text{mV}$, $[\text{K}^+]_{\text{out}} = 4\text{mM}$ and $[\text{K}^+]_{\text{in}} = 70\text{mM}$. These parameters yield an $\text{EDF}_{\text{K}^+} = 54\text{mV}$, corresponding to $\sim 5.2\text{kJ/mol}$ of free energy available for K^+ -powered cholesterol transport by PTCH1. To put these free energy values in perspective, we consider the Na^+ -powered glucose symporter SGLT2 (Coady et al., 2017) and the Na^+ -powered choline symporter hCHT (Iwamoto et al., 2006). These proteins transport one Na^+ ion per substrate molecule, and voltage-current studies show that an $\text{EDF}_{\text{Na}^+} > 5\text{kJ/mol Na}^+$ is sufficient to power these transporters. Thus, the energy supplied by the plasma membrane Na^+ gradient, or by the ciliary K^+ gradient is likely sufficient to power substrate transport by DISP1 and PTCH1, respectively.

STAR Methods

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for reagents should be directed to, and will be fulfilled by, the Lead Contact, Adrian Salic (asalic@hms.harvard.edu).

Materials Availability—All newly generated plasmids and cell lines are available from the Lead Contact upon request.

Data and Code Availability—This study did not generate datasets. Custom MATLAB scripts utilized for image analysis are available from the Lead Contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell culture—HEK293T, HEK293S GnT1[−], NIH-3T3 and L cells were purchased from ATCC. L cells, HEK293T and HEK293S GnT1[−] cells, and *Ptch1*^{−/−} MEFs (Goodrich et al., 1997) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), penicillin, and streptomycin. NIH-3T3 cells were grown in DMEM with 10% bovine calf serum (BCS), penicillin, and streptomycin. HEK293T and HEK293S GnT1[−] cells are female. NIH-3T3 and L cells are male. The sex of *Ptch1*^{−/−} MEFs has not been determined. Stable lines were generated by lentiviral transduction, followed by blasticidin selection (50 $\mu\text{g/mL}$), as described (Tukachinsky et al., 2012). Briefly, HEK293T cells were transiently transfected (TransIT-293, Mirus) with lentiviral constructs bearing transgenes of interest; lentivirus-conditioned medium was harvested after 48 h and used to infect cells of interest. For transient expression experiments, cells were transfected using polyethylenimine (PEI, Polysciences). For SHH-NL5 titration experiments, the amount of transfected DNA was equalized by mixing the SHH-NL5 expression construct with carrier plasmid DNA (pBluescript).

Generation of DISP1 knockout (DISP1^{KO}) HEK293T cells by CRISPR/Cas9—

Two gRNA sequences were chosen, in exons 2 and 4 (the first and third exons with coding sequence) of human *DISP1* (crispr.mit.edu). Targeting constructs were generated in a derivative of pX330 (Cong et al., 2013) expressing EGFP-tagged Cas9. HEK293T cells were co-transfected with the two targeting plasmids, and EGFP-positive cells, isolated by fluorescence-activated sorting, were plated clonally. Genomic DNA was extracted from single clones, and was initially screened by PCR, to identify cells with large deletions spanning the two gRNA-targeted loci. A clonal line harboring a 47.7-kbp deletion allele was identified. Both gRNA loci were then PCR amplified and Sanger sequenced to define all lesions present. Lesions were further confirmed by sequencing target locus amplicons on a MiSeq instrument (Illumina). The DISP1^{KO} line used in this study harbors a deletion allele, as well as alleles with frameshifts at both targeted loci. Two indels causing frameshifts were identified at the exon 2 locus, and one indel causing frameshift at the exon 4 locus. No wild-type sequences were observed by MiSeq at either locus. That two frameshifts were identified for exon 2 perhaps reflects the presence of 3 copies of the *DISP1* locus in HEK293T cells.

METHOD DETAILS

Antibodies—The following antibodies were purchased: rabbit anti-SHH monoclonal antibody (Cell Signaling Technology), rabbit anti-WNT3A monoclonal antibody (Cell Signaling Technology), rabbit anti-GFP polyclonal antibody (Rockland), mouse monoclonal anti-tubulin (Sigma), mouse anti-acetylated tubulin monoclonal antibody (Sigma), rat anti-HA–HRP conjugate (Roche), sheep anti-mouse IgG–HRP conjugate (Jackson ImmunoResearch), donkey anti-rabbit IgG–HRP conjugate (GE Healthcare), donkey anti-goat IgG–Alexa Fluor 488 conjugate (Thermo), donkey anti-rabbit IgG–Alexa Fluor 555 conjugate (Thermo), and donkey anti-chicken IgY–Alexa Fluor 647 conjugate (Jackson ImmunoResearch). Antibodies against mCherry (Nedelcu et al., 2013) and against mouse SMO (Tukachinsky et al., 2010) were described previously. Polyclonal antibodies against mouse ARL13B (mARL13B), a marker of cilia, were raised in chickens. Briefly, 6xHis-tagged mARL13B was expressed in *E. coli* (BL21 DE3 pLysS, Novagen), was purified as a soluble protein, and was used to immunize hens (Abcore). Whole IgY was isolated from eggs laid by the immunized hens, as described (Pauly et al., 2011). Anti-mARL13B antibodies were isolated from whole IgY, by affinity purification against recombinant MBP-tagged mARL13B immobilized on Affi-Gel 10 beads (Bio-Rad).

DNA constructs—All constructs were subcloned into a lentiviral CMV promoter-driven expression vector. Overlapping PCR was used to introduce mutations and to assemble fusions, and the resulting constructs were confirmed by sequencing. Mouse DISP1 and PTCH1 were tagged with mCherry fluorescent protein at their C-termini. SHH-NL fusions were generated by inserting the coding sequence for NanoLuc (NL, Promega), flanked by two glycine residues on either end, between the following residues of the N-terminal signaling domain of human SHH: K32 and R33; N69 and S70; S70 and E71; P82 and D83; N91 and T92; P118 and G119; E130 and D131; R153 and D154; K194 and S195. WNT3A-NL consists of the influenza hemagglutinin signal sequence, one copy of the FLAG epitope, the NL sequence, followed by the sequence of mouse WNT3A without signal sequence; this is the same tagging location as used to generate epitope-tagged functional WNT3A (Farin et

al., 2016). The expression construct for producing mouse SCUBE2 contained, from the N-terminus: the influenza hemagglutinin signal sequence, one copy of the FLAG epitope, the mutant bacterial dehalogenase HaloTag7 (Ohana et al., 2009), a PreScission protease cleavage site, and the sequence of SCUBE2 without the signal sequence. To increase the yield of full-length SCUBE2, the putative furin cleavage site was mutated (R274A, R276A, R277A).

Custom media formulations—Cell culture media of defined ionic composition were based on Tyrode's solution, and are described in Table S1.

Fluorescence-activated cell sorting—A single cell suspension at 10×10^6 cells per mL in phenol red-free DMEM, supplemented with 0.5% FBS was made by trypsinizing, pelleting and resuspending cells. The suspension was passed through a 35 μ m nylon strainer and cells were sorted on a FACS Aria III (BD Biosciences) cell sorter equipped with a 561nm yellow laser and multi-sort option. The population fluorescence distribution was measured for each population after the sort.

Protein expression and purification—SCUBE2 was affinity purified on anti-FLAG-M1 resin (Millipore), from media conditioned by HEK293S GnT1⁻ cells stably transduced with the SCUBE2-expressing lentivirus. Briefly, conditioned media was clarified by centrifugation and filtration, and was then adjusted to 300 mM NaCl, 2 mM CaCl₂ and 0.1% Triton X-100. The media was applied twice to a column packed with anti-FLAG-M1 resin, at 4°C. The column was washed with >10 column volumes of wash buffer with detergent (10 mM Tris pH 8, 150 mM NaCl, 2mM CaCl₂, 0.1% Triton X-100), followed by >20 column volumes of wash buffer without detergent (10 mM Tris pH 8, 150 mM NaCl, 2mM CaCl₂). The protein was eluted at room temperature, with 100 μ g/mL FLAG peptide (Genscript) in Tris-buffered saline (TBS, 10 mM Tris pH 8, 150 mM NaCl). Fractions containing the protein were pooled and concentrated using centrifugal concentrators (Millipore), according to manufacturer's instructions. SCUBE2 was further purified by gel filtration on a Superdex 200 column (GE Healthcare).

Luciferase-based SHH release assays—HEK293T cells stably expressing SHH-NL5 were plated on poly-D-lysine, in complete media. The next day, the cells were exchanged into DMEM, and were cultured overnight. The cells were washed three times with DMEM, to remove all serum, and were preincubated with cycloheximide (100 μ g/mL, Sigma) for 30 minutes. The release reaction was started by switching the cells into media supplemented with purified SCUBE2, BSA (negative control), or methyl- β -cyclodextrin (M β CD, Sigma), in the continued presence of cycloheximide. The cells were incubated at 37°C in a cell culture incubator, and aliquots of the supernatant were withdrawn at specified time points. Between time points, the plates were gently shaken to ensure even mixing of media. Collected supernatants were centrifuged, to remove possible cellular debris, and NanoLuc activity was measured on a Wallac Victor3 Microplate Reader (Perkin-Elmer), using Nano-Glo Luciferase Assay Substrate (Promega), according to the manufacturer's instructions. Luminescence measurements were corrected for the decreasing supernatant volume during the assay. Only linear portions of release curves were used to calculate initial release rates.

In experiments comparing SHH-NL5 release between different cell lines, the cells were also harvested at the end of the experiment (30 minutes), were lysed in TBS with 0.2% Triton X-100, and NanoLuc activity was measured, to determine SHH-NL5 expression levels. Only a small fraction (<5%) of total SHH-NL5 was released from cells during the 30-minute duration of the release assay. Specific modifications of this protocol used during initial assay development are noted in the associated figure legends. Luminescence dynamic range and standard curves were determined using recombinant NanoLuc, expressed and purified from bacteria.

WNT3A release assays—L cells stably expressing WNT3A or WNT3A-NL were grown overnight in complete media, and were washed thrice with DMEM. The cells were then incubated overnight in DMEM without serum or with 10% FBS, in the absence or presence of the Porcupine (PORCN) inhibitor, IWP-2 (2 μ M, Sigma). Media was harvested, was centrifuged to remove cellular debris, and released WNT3A was detected by SDS-PAGE and immunoblotting, while released WNT3A-NL was measured by luminescence, as for SHH-NL5. Total WNT3A-NL was measured in cell lysates, as for SHH-NL5. For measuring the effect of the Na⁺ gradient on WNT3A-NL release rates, after washing with DMEM, the cells were preincubated in DMEM with cycloheximide (100 μ g/mL) for 30 minutes, after which WNT3A-NL release was initiated by switching cells into the indicated Tyrode's media, supplemented with cycloheximide and 2% FBS. Aliquots of the supernatant were withdrawn at specified time points, and released WNT3A-NL was measured as above. Remaining WNT3A-NL was measured in cell lysates.

Total protein measurements—Total protein was measured using a BCA assay (G Biosciences), according to manufacturer's recommendations, using cell lysates harvested at the end of each experiment (see above). Briefly, 5–10 μ L of cell lysate was combined with 1mL BCA reagent and incubated at 37°C for 60min. Absorbance was read at 562nm using a Cary 300 UV-Vis spectrophotometer (Agilent). Protein concentration was determined according to a BSA standard curve.

Immunoblot-based SHH release—On day 1, wild-type or DISP1^{KO} HEK293T cells were plated in 6-well format, such that they would reach 50% confluence the next day. On day 2, cells were transiently transfected with SHH and the indicated SCUBE2 and DISP1 constructs. On day 3, the cells were washed three times with DMEM at 2-hour intervals to remove serum and then into DMEM for overnight collection. Conditioned media was harvested, centrifuged to remove debris, and then subjected to trichloroacetic acid (TCA) precipitation. Precipitated proteins were separated by SDS-PAGE, and released SHH was detected by immunoblotting (Tukachinsky et al., 2012).

Microscopy and immunofluorescence—On day 1, cells were plated on gelatin-coated glass coverslips, such that they would reach confluence the next day. On day 2, the cells were serum-starved, by overnight incubation in DMEM. On day 3, the cells were incubated under the indicated conditions, after which they were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, 2.7mM KCl, 137mM NaCl) at room temperature, for 15 minutes. The cells were washed with TBS, were

permeabilized with TBST (TBS with 0.2% Triton X-100), and were incubated in blocking solution (25mg/mL BSA in TBST). Immunofluorescence was performed as described (Tukachinsky et al., 2010). The primary antibodies were: chicken anti-ARL13B, rabbit anti-mCherry, goat anti-SMO, each at 2µg/mL final concentration. The secondary antibodies were: donkey anti-goat IgG–Alexa Fluor 488 conjugate, donkey anti-rabbit IgG–Alexa Fluor 555 conjugate, donkey anti-chicken IgY–Alexa Fluor 647, each at 1µg/mL final concentration. Images were acquired with a 40x Plan Apo 0.95 N.A. objective lens (Nikon), on a TE2000E microscope (Nikon) equipped with motorized stage (Prior Scientific) and an OrcaER camera (Hamamatsu Photonics). Ciliary fluorescence intensity was quantified using custom-built scripts implemented in MATLAB, as described (Nedelcu et al., 2013). At least 100 cilia were analyzed per condition. Details of statistical testing are described in figure legends.

Quantitative RT-PCR—Measurements of *Gli1* expression, relative to the housekeeping gene *Rpl27*, were done by quantitative RT-PCR, as described (Tukachinsky et al., 2016). Briefly, cells were serum-starved overnight and treated as described in the figure legends. TRIzol (Thermo) was used to extract total RNA, which was treated with DNase I (Promega) and re-extracted with TRIzol prior to reverse transcription (LunaScript RT SuperMix, NEB). cDNA samples were quantified with PowerUp SYBR Green Master Mix (Thermo) and the indicated primers (Key Resource Table). Biological triplicates were performed for all assays.

E_m measurements—DiBAC₄(3) (Sigma), a dye with E_m-sensitive uptake (Epps et al., 1994), was used to measure E_m for cells incubated in various media. Confluent NIH-3T3 cells were serum-starved in DMEM for 4–6 hours, after which the cells were detached by trypsinization, were washed, and the cell suspension was incubated with DiBAC₄(3) (400nM), for 30 minutes at 37°C. The cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences), using 488nm laser excitation and a 530/30 emission filter. Media with various K⁺ concentrations were used as positive controls for membrane depolarization. A standard curve for DiBAC₄(3) fluorescence was determined by permeabilizing cells with 2µg/mL gramicidin A (Sigma), incubating them with DiBAC₄(3) (0, 25, 50, 100, 200, 400nM), followed by fluorescence measurement by flow cytometry.

Cellular ATP measurements—NIH-3T3 cells were plated in 96-well plates, at a density of 40,000 cells per well. The next day, the complete media was replaced with DMEM, followed by overnight incubation. The cells were washed again with DMEM and were then incubated in media of defined ionic composition, for 3 or 6 hours. After the incubation period, cellular ATP was measured using the CellTiter-Glo reagent (Promega), according to manufacturer's instructions, using a Wallac Victor3 Microplate Reader. As control, cellular ATP was depleted by incubation in PBS supplemented with CCCP (1µM or 5µM, as indicated; Sigma), staurosporine (2µM, LC Laboratories), or oligomycin A (100nM, Sigma). Standard curves were determined using pure ATP solutions. All ATP measurements were performed at least three times.

Cellular NAD(P)H measurements—NAPD(P)H was assayed using an MTT viability assay (Cayman Chemical). HEK293T cells were plated on poly-D-lysine coated 96-well

plates, at a density of 50,000 cells per well. The next day, cells were gently washed with DMEM (0.1mL/well) and were incubated in test media for 3 hours. Media was then removed and PBS (0.1mL/well) and MTT reagent (5μL/well) were added, followed by incubation at 37°C for 3 hours. Dissolution solution (0.1mL/well) was then added, followed by incubation at room temperature overnight. The next day, absorbance at 570nm was read using a microplate reader.

Kinetic model for DISP1-catalyzed SHH release—We hypothesized that DISP1 acts as an enzyme in SHH release. This scenario is described by the following scheme:

SHH and SCUBE2 (added purified protein) are substrates for DISP1, while SCUBE2:SHH (i.e. released SHH) is the reaction product. We apply an equilibrium assumption, that binding is faster than enzyme kinetics, which means that we can describe the concentrations of intermediate species according to their binding equilibria:

$$[\text{SHH:DISP1}] = \frac{[\text{SHH}][\text{DISP1}]}{K_{d1}}$$

$$[\text{SCUBE2:DISP1}] = \frac{[\text{SCUBE2}][\text{DISP1}]}{K_{d2}}$$

The ternary complex SHH:SCUBE2:DISP1 can form either by SCUBE2 joining SHH:DISP1, or by SHH joining SCUBE2:DISP1, hence two Michaelis–Menten constants, K_{M1} and K_{M2} , respectively. Importantly, the rate of SHH release (v_{release}) does not depend on the order of substrate association with DISP1. Putting everything together, we obtain:

$$V_{\text{release}} = \frac{V_{\text{max}}}{1 + \frac{K_{M1}}{[\text{SCUBE2}]} + \frac{K_{M2}}{[\text{SHH}]} + \frac{K_{d1}K_{M2}}{[\text{SCUBE2}][\text{SHH}]}} \quad [1]$$

where $V_{\text{max}} = [\text{DISP1}] * k_{\text{cat}}$.

Rearranging equation 1 to show the release rate dependence on $[\text{SCUBE2}]$, we have:

$$V_{\text{release}} = \frac{V_{\text{max}}[\text{SCUBE2}]}{K_M + [\text{SCUBE2}](1 + \frac{K_{M2}}{[\text{SHH}]})} \quad [2]$$

where $K_M = K_{M1} + \frac{K_{d1}K_{M2}}{[\text{SHH}]}$.

In most of our experiments, SHH is saturating, i.e. $[\text{SHH}] \gg K_{M2}$. This yields equation 3:

$$V_{\text{release}} = \frac{V_{\text{max}}[\text{SCUBE2}]}{K_{M1} + [\text{SCUBE2}]} \quad [3]$$

Equation 3 predicts that the release rate will saturate as a function of [SCUBE2], which is what is observed (Fig.2E). This shows that SHH release by SCUBE2 is a catalyzed process, as opposed to a simple binding equilibrium.

According to equation 3, if DISP1 were the enzyme catalyzing SHH release, then V_{max} , but not K_M , would be a function of [DISP1], since:

$$V_{\text{max}} = [\text{DISP1}] * k_{\text{cat}}$$

We test this prediction by varying [DISP1] (Fig.2F–H, Fig.2I–K, and Fig.S2H–J), and we find that DISP1 levels indeed affect V_{max} , but not K_M , which remains constant at 2–3 μM across multiple independent experiments. These results strongly suggest that DISP1 is the rate-limiting enzyme that catalyzes SHH release.

Kinetic model for DISP1 playing an accessory role in SHH release—As an alternative hypothesis, we considered a second scheme, wherein DISP1 functions as an adaptor between membrane-bound SHH and an unknown enzyme (E) catalyzing SCUBE2:SHH release. This scenario is described by the following scheme:

Applying an equilibrium assumption as above, we obtain the following:

$$[\text{SHH:DISP1}] = \frac{[\text{SHH}][\text{DISP1}]}{K_{di}}$$

$$[\text{SHH:DISP1:E}] = \frac{[\text{SHH:DISP1}][E]}{K_{d1}} = \frac{[\text{SHH}][\text{DISP1}][E]}{K_{di}K_{d1}}$$

$$\text{SCUBE2:E} = \frac{[\text{SCUBE2}][E]}{K_{d2}}$$

Michaelis–Menten constants are similar to those above, with K_{M1} describing SCUBE2 joining the enzyme:substrate complex and K_{M2} describing SHH:DISP1 joining the enzyme substrate complex; k_{cat} is the catalytic constant of the enzyme E. The resulting rate equation is:

$$V_{\text{release}} = \frac{V_{\text{max}}}{1 + \frac{K_{M1}}{[\text{SCUBE2}]} + \frac{K_{M2}K_{di}}{[\text{SHH}][\text{DISP1}]} + \frac{K_{d1}K_{M2}K_{di}}{[\text{SCUBE2}][\text{SHH}][\text{DISP1}]}} \quad [4]$$

where $V_{\text{max}} = [E] * k_{\text{cat}}$.

Rearranging equation 4 to show the release rate dependence on [SCUBE2], we have:

$$V_{\text{release}} = \frac{V_{\text{max}}[\text{SCUBE2}]}{K_M^* + [\text{SCUBE2}](1 + \frac{K_{M2}K_{di}}{[\text{SHH}][\text{DISP1}]})} \quad [5]$$

where $K_M^* = K_{M1} + \frac{K_{d1}K_{M2}K_{di}}{[\text{SHH}][\text{DISP1}]}$.

This scenario also predicts release rate saturation as a function of SCUBE2; however, now the measured K_M (K_M^*), but not V_{max} , would be a function of [DISP1]. This is the opposite of what we observe experimentally, indicating that DISP1 itself, not another enzyme, catalyzes SHH release.

Calculations of electrochemical driving force (EDF)—The EDF for Na^+ and K^+ for the experiments in Fig.3E, F was calculated using equation E1:

$$\text{EDF}_{\text{Na}^+} = E_m - \frac{RT}{F} \ln \left(\frac{[\text{Na}^+]_{\text{out}}}{[\text{Na}^+]_{\text{in}}} \right) \quad (\text{E1})$$

where R is the universal gas constant, $T=300\text{K}$, F is the Faraday constant, and E_m is the resting membrane potential. E_m was estimated using the Goldman–Hodgkin–Katz equation, assuming a ratio of 10:1 in $\text{Na}^+:\text{K}^+$ permeability, $[\text{Na}^+]_{\text{in}} = 15\text{mM}$, $[\text{K}^+]_{\text{in}}=125 \text{ mM}$, $[\text{Cl}^-]_{\text{in}}=15 \text{ mM}$.

Blot image processing—Blot images in which species of different molecular weights were probed simultaneously were cropped in Photoshop CS5. Where necessary, such blots were resized along the horizontal axis to present clear vertical alignment of lanes across multiple probed species. Original images are available upon request.

QUANTIFICATION AND STATISTICAL ANALYSIS

SHH release was measured as a single measurement over multiple time points, unless indicated otherwise in the figure legends. Release rate was calculated as the slope of the linear regression line fit to the linear portion, typically 1–30min, of the SHH release data. Curve fitting was performed in Prism 8 (GraphPad Software), using least-squares linear or nonlinear regression, as indicated in the figure legends. Parameter estimates are reported with associated standard errors of the estimate. Statistical tests and associated significance threshold are reported in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH grants R01 GM122920 and GM135262 (to A.S.), and by NIH predoctoral fellowship F31GM120833 (to B.M.W.). We thank Marie Bao for reading the manuscript and for helpful suggestions, and members of the Salic lab for discussions.

References

- Aires JR, and Nikaido H (2005). Aminoglycosides are captured from both periplasm and cytoplasm by the AcrD multidrug efflux transporter of *Escherichia coli*. *J Bacteriol* 187, 1923–1929. [PubMed: 15743938]
- Alcedo J, Ayzenzon M, Von Ohlen T, Noll M, and Hooper JE (1996). The *Drosophila* *smoothed* gene encodes a seven-pass membrane protein, a putative receptor for the hedgehog signal. *Cell* 86, 221–232. [PubMed: 8706127]
- Amanai K, and Jiang J (2001). Distinct roles of Central missing and Dispatched in sending the Hedgehog signal. *Development* 128, 5119–5127. [PubMed: 11748147]
- Blose SH, Meltzer DI, and Feramisco JR (1984). 10-nm filaments are induced to collapse in living cells microinjected with monoclonal and polyclonal antibodies against tubulin. *The Journal of cell biology* 98, 847–858. [PubMed: 6538204]
- Burke R, Nellen D, Bellotto M, Hafen E, Senti KA, Dickson BJ, and Basler K (1999). Dispatched, a novel sterol-sensing domain protein dedicated to the release of cholesterol-modified hedgehog from signaling cells. *Cell* 99, 803–815. [PubMed: 10619433]
- Byrne EF, Sircar R, Miller PS, Hedger G, Luchetti G, Nachtergaele S, Tully MD, Mydock-McGrane L, Covey DF, Rambo RP, et al. (2016). Structural basis of Smoothed regulation by its extracellular domains. *Nature* 535, 517–522. [PubMed: 27437577]
- Carstea ED, Morris JA, Coleman KG, Loftus SK, Zhang D, Cummings C, Gu J, Rosenfeld MA, Pavan WJ, Krizman DB, et al. (1997). Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* 277, 228–231. [PubMed: 9211849]
- Caspary T, Garcia-Garcia MJ, Huangfu D, Eggenschwiler JT, Wyler MR, Rakeman AS, Alcorn HL, and Anderson KV (2002). Mouse Dispatched homolog1 is required for long-range, but not juxtacrine, Hh signaling. *Current biology : CB* 12, 1628–1632. [PubMed: 12372258]
- Chamoun Z, Mann RK, Nellen D, von Kessler DP, Bellotto M, Beachy PA, and Basler K (2001). Skinny hedgehog, an acyltransferase required for palmitoylation and activity of the hedgehog signal. *Science* 293, 2080–2084. [PubMed: 11486055]
- Chang DT, Lopez A, von Kessler DP, Chiang C, Simandl BK, Zhao R, Seldin MF, Fallon JF, and Beachy PA (1994). Products, genetic linkage and limb patterning activity of a murine hedgehog gene. *Development* 120, 3339–3353. [PubMed: 7720571]
- Chen JK, Taipale J, Young KE, Maiti T, and Beachy PA (2002). Small molecule modulation of Smoothed activity. *Proceedings of the National Academy of Sciences of the United States of America* 99, 14071–14076. [PubMed: 12391318]
- Coady MJ, Wallendorff B, and Lapointe JY (2017). Characterization of the transport activity of SGLT2/MAP17, the renal low-affinity Na(+)-glucose cotransporter. *Am J Physiol Renal Physiol* 313, F467–F474. [PubMed: 28592437]
- Cong L, Ran FA, Cox D, Lin S, Barretto R, Habib N, Hsu PD, Wu X, Jiang W, Marraffini LA, et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. [PubMed: 23287718]
- Cooper MK, Wassif CA, Krakowiak PA, Taipale J, Gong R, Kelley RI, Porter FD, and Beachy PA (2003). A defective response to Hedgehog signaling in disorders of cholesterol biosynthesis. *Nat Genet* 33, 508–513. [PubMed: 12652302]

- Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, and Reiter JF (2005). Vertebrate Smoothed functions at the primary cilium. *Nature* 437, 1018–1021. [PubMed: 16136078]
- Courtney KC, Fung KY, Maxfield FR, Fairn GD, and Zha X (2018). Comment on ‘Orthogonal lipid sensors identify transbilayer asymmetry of plasma membrane cholesterol’. *eLife* 7.
- Creanga A, Glenn TD, Mann RK, Saunders AM, Talbot WS, and Beachy PA (2012). Scube/You activity mediates release of dually lipid-modified Hedgehog signal in soluble form. *Genes & development* 26, 1312–1325. [PubMed: 22677548]
- Davis HR Jr., Zhu LJ, Hoos LM, Tetzloff G, Maguire M, Liu J, Yao X, Iyer SP, Lam MH, Lund EG, et al. (2004). Niemann-Pick C1 Like 1 (NPC1L1) is the intestinal phytosterol and cholesterol transporter and a key modulator of whole-body cholesterol homeostasis. *The Journal of biological chemistry* 279, 33586–33592. [PubMed: 15173162]
- Deffieu MS, and Pfeffer SR (2011). Niemann-Pick type C 1 function requires luminal domain residues that mediate cholesterol-dependent NPC2 binding. *Proceedings of the National Academy of Sciences of the United States of America* 108, 18932–18936. [PubMed: 22065762]
- Delling M, DeCaen PG, Doerner JF, Febvay S, and Clapham DE (2013). Primary cilia are specialized calcium signalling organelles. *Nature* 504, 311–314. [PubMed: 24336288]
- Delmar JA, Su CC, and Yu EW (2015). Heavy metal transport by the CusCFBA efflux system. *Protein Sci* 24, 1720–1736. [PubMed: 26258953]
- Echelard Y, Epstein DJ, St-Jacques B, Shen L, Mohler J, McMahon JA, and McMahon AP (1993). Sonic hedgehog, a member of a family of putative signaling molecules, is implicated in the regulation of CNS polarity. *Cell* 75, 1417–1430. [PubMed: 7916661]
- Epps DE, Wolfe ML, and Groppi V (1994). Characterization of the steady-state and dynamic fluorescence properties of the potential-sensitive dye bis-(1,3-dibutylbarbituric acid)trimethine oxonol (Dibac4(3)) in model systems and cells. *Chemistry and physics of lipids* 69, 137–150. [PubMed: 8181103]
- Farin HF, Jordens I, Mosa MH, Basak O, Korving J, Tauriello DV, de Punder K, Angers S, Peters PJ, Maurice MM, et al. (2016). Visualization of a short-range Wnt gradient in the intestinal stem-cell niche. *Nature* 530, 340–343. [PubMed: 26863187]
- Gong X, Qian H, Cao P, Zhao X, Zhou Q, Lei J, and Yan N (2018). Structural basis for the recognition of Sonic Hedgehog by human Patched!. *Science* 361.
- Goodrich LV, Milenkovic L, Higgins KM, and Scott MP (1997). Altered neural cell fates and medulloblastoma in mouse patched mutants. *Science* 277, 1109–1113. [PubMed: 9262482]
- Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, Otto P, Zimmerman K, Vidugiris G, Machleidt T, et al. (2012). Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol* 7, 1848–1857. [PubMed: 22894855]
- Hollway GE, Maule J, Gautier P, Evans TM, Keenan DG, Lohs C, Fischer D, Wicking C, and Currie PD (2006). Scube2 mediates Hedgehog signalling in the zebrafish embryo. *Developmental biology* 294, 104–118. [PubMed: 16626681]
- Huang P, Nedelcu D, Watanabe M, Jao C, Kim Y, Liu J, and Salic A (2016). Cellular Cholesterol Directly Activates Smoothed in Hedgehog Signaling. *Cell* 166, 1176–1187 e1114. [PubMed: 27545348]
- Humke EW, Dorn KV, Milenkovic L, Scott MP, and Rohatgi R (2010). The output of Hedgehog signaling is controlled by the dynamic association between Suppressor of Fused and the Gli proteins. *Genes & development* 24, 670–682. [PubMed: 20360384]
- Infante RE, Wang ML, Radhakrishnan A, Kwon HJ, Brown MS, and Goldstein JL (2008). NPC2 facilitates bidirectional transfer of cholesterol between NPC1 and lipid bilayers, a step in cholesterol egress from lysosomes. *Proceedings of the National Academy of Sciences of the United States of America* 105, 15287–15292. [PubMed: 18772377]
- Ingham PW, and McMahon AP (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes & development* 15, 3059–3087. [PubMed: 11731473]
- Ingham PW, Nystedt S, Nakano Y, Brown W, Stark D, van den Heuvel M, and Taylor AM (2000). Patched represses the Hedgehog signalling pathway by promoting modification of the Smoothed protein. *Current biology : CB* 10, 1315–1318. [PubMed: 11069117]

- Iwamoto H, Blakely RD, and De Felice LJ (2006). Na⁺, Cl⁻, and pH dependence of the human choline transporter (hCHT) in *Xenopus* oocytes: the proton inactivation hypothesis of hCHT in synaptic vesicles. *J Neurosci* 26, 9851–9859. [PubMed: 17005849]
- Kawakami A, Nojima Y, Toyoda A, Takahoko M, Satoh M, Tanaka H, Wada H, Masai I, Terasaki H, Sakaki Y, et al. (2005). The zebrafish-secreted matrix protein you/scube2 is implicated in long-range regulation of hedgehog signaling. *Current biology : CB* 15, 480–488. [PubMed: 15753045]
- Kawakami T, Kawcak T, Li YJ, Zhang W, Hu Y, and Chuang PT (2002). Mouse dispatched mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling. *Development* 129, 5753–5765. [PubMed: 12421714]
- Kilambi KP, and Gray JJ (2012). Rapid calculation of protein pKa values using Rosetta. *Biophysical journal* 103, 587–595. [PubMed: 22947875]
- Krauss S, Concordet JP, and Ingham PW (1993). A functionally conserved homolog of the *Drosophila* segment polarity gene *hh* is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* 75, 1431–1444. [PubMed: 8269519]
- Lee HC (1984a). A membrane potential-sensitive Na⁺-H⁺ exchange system in flagella isolated from sea urchin spermatozoa. *The Journal of biological chemistry* 259, 15315–15319. [PubMed: 6096367]
- Lee HC (1984b). Sodium and proton transport in flagella isolated from sea urchin spermatozoa. *The Journal of biological chemistry* 259, 4957–4963. [PubMed: 6325412]
- Luchetti G, Sircar R, Kong JH, Nachtergaele S, Sagner A, Byrne EF, Covey DF, Siebold C, and Rohatgi R (2016). Cholesterol activates the G-protein coupled receptor Smoothened to promote Hedgehog signaling. *eLife* 5.
- Lum L, and Beachy PA (2004). The Hedgehog response network: sensors, switches, and routers. *Science* 304, 1755–1759. [PubMed: 15205520]
- Ma Y, Erkner A, Gong R, Yao S, Taipale J, Basler K, and Beachy PA (2002). Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. *Cell* 111, 63–75. [PubMed: 12372301]
- Murakami S, Nakashima R, Yamashita E, Matsumoto T, and Yamaguchi A (2006). Crystal structures of a multidrug transporter reveal a functionally rotating mechanism. *Nature* 443, 173–179. [PubMed: 16915237]
- Myers BR, Neahring L, Zhang Y, Roberts KJ, and Beachy PA (2017). Rapid, direct activity assays for Smoothened reveal Hedgehog pathway regulation by membrane cholesterol and extracellular sodium. *Proceedings of the National Academy of Sciences of the United States of America* 114, E11141–E11150. [PubMed: 29229834]
- Myers BR, Sever N, Chong YC, Kim J, Belani JD, Rychnovsky S, Bazan JF, and Beachy PA (2013). Hedgehog pathway modulation by multiple lipid binding sites on the smoothened effector of signal response. *Developmental cell* 26, 346–357. [PubMed: 23954590]
- Nachtergaele S, Whalen DM, Mydock LK, Zhao Z, Malinauskas T, Krishnan K, Ingham PW, Covey DF, Siebold C, and Rohatgi R (2013). Structure and function of the Smoothened extracellular domain in vertebrate Hedgehog signaling. *eLife* 2, e01340. [PubMed: 24171105]
- Nakano Y, Guerrero I, Hidalgo A, Taylor A, Whittle JR, and Ingham PW (1989). A protein with several possible membrane-spanning domains encoded by the *Drosophila* segment polarity gene *patched*. *Nature* 341, 508–513. [PubMed: 2797178]
- Nakano Y, Kim HR, Kawakami A, Roy S, Schier AF, and Ingham PW (2004). Inactivation of dispatched 1 by the chameleon mutation disrupts Hedgehog signalling in the zebrafish embryo. *Developmental biology* 269, 381–392. [PubMed: 15110707]
- Nedelcu D, Liu J, Xu Y, Jao C, and Salic A (2013). Oxysterol binding to the extracellular domain of Smoothened in Hedgehog signaling. *Nature chemical biology* 9, 557–564. [PubMed: 23831757]
- Nikaido H (2018). RND transporters in the living world. *Res Microbiol* 169, 363–371. [PubMed: 29577985]
- Ohana RF, Encell LP, Zhao K, Simpson D, Slater MR, Urh M, and Wood KV (2009). HaloTag7: a genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. *Protein Expr Purif* 68, 110–120. [PubMed: 19464373]

- Pauly D, Chacana PA, Calzado EG, Brembs B, and Schade R (2011). IgY technology: extraction of chicken antibodies from egg yolk by polyethylene glycol (PEG) precipitation. *J Vis Exp*.
- Pepinsky RB, Zeng C, Wen D, Rayhorn P, Baker DP, Williams KP, Bixler SA, Ambrose CM, Garber EA, Miatkowski K, et al. (1998). Identification of a palmitic acid-modified form of human Sonic hedgehog. *The Journal of biological chemistry* 273, 14037–14045. [PubMed: 9593755]
- Porter JA, Young KE, and Beachy PA (1996). Cholesterol modification of hedgehog signaling proteins in animal development. *Science* 274, 255–259. [PubMed: 8824192]
- Qi X, Schmiede P, Coutavas E, and Li X (2018a). Two Patched molecules engage distinct sites on Hedgehog yielding a signaling-competent complex. *Science* 362.
- Qi X, Schmiede P, Coutavas E, Wang J, and Li X (2018b). Structures of human Patched and its complex with native palmitoylated sonic hedgehog. *Nature* 560, 128–132. [PubMed: 29995851]
- Qian H, Cao P, Hu M, Gao S, Yan N, and Gong X (2019). Inhibition of tetrameric Patched1 by Sonic Hedgehog through an asymmetric paradigm. *Nat Commun* 10, 2320. [PubMed: 31127104]
- Reeves PJ, Callewaert N, Contreras R, and Khorana HG (2002). Structure and function in rhodopsin: high-level expression of rhodopsin with restricted and homogeneous N-glycosylation by a tetracycline-inducible N-acetylglucosaminyltransferase I-negative HEK293S stable mammalian cell line. *Proceedings of the National Academy of Sciences of the United States of America* 99, 13419–13424. [PubMed: 12370423]
- Riddle RD, Johnson RL, Laufer E, and Tabin C (1993). Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75, 1401–1416. [PubMed: 8269518]
- Rohatgi R, Milenkovic L, and Scott MP (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317, 372–376. [PubMed: 17641202]
- Rubin LL, and de Sauvage FJ (2006). Targeting the Hedgehog pathway in cancer. *Nat Rev Drug Discov* 5, 1026–1033. [PubMed: 17139287]
- Seeger MA, Schiefner A, Eicher T, Verrey F, Diederichs K, and Pos KM (2006). Structural asymmetry of AcrB trimer suggests a peristaltic pump mechanism. *Science* 313, 1295–1298. [PubMed: 16946072]
- Su CC, Li M, Gu R, Takatsuka Y, McDermott G, Nikaido H, and Yu EW (2006). Conformation of the AcrB multidrug efflux pump in mutants of the putative proton relay pathway. *J Bacteriol* 188, 7290–7296. [PubMed: 17015668]
- Taipale J, Cooper MK, Maiti T, and Beachy PA (2002). Patched acts catalytically to suppress the activity of Smoothened. *Nature* 418, 892–897. [PubMed: 12192414]
- Takatsuka Y, and Nikaido H (2006). Threonine-978 in the transmembrane segment of the multidrug efflux pump AcrB of *Escherichia coli* is crucial for drug transport as a probable component of the proton relay network. *J Bacteriol* 188, 7284–7289. [PubMed: 17015667]
- Tukachinsky H, Kuzmickas RP, Jao CY, Liu J, and Salic A (2012). Dispatched and Scube mediate the efficient secretion of the cholesterol-modified hedgehog ligand. *Cell reports* 2, 308–320. [PubMed: 22902404]
- Tukachinsky H, Lopez LV, and Salic A (2010). A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. *The Journal of cell biology* 191, 415–428. [PubMed: 20956384]
- Tukachinsky H, Petrov K, Watanabe M, and Salic A (2016). Mechanism of inhibition of the tumor suppressor Patched by Sonic Hedgehog. *Proceedings of the National Academy of Sciences of the United States of America* 113, E5866–E5875. [PubMed: 27647915]
- van den Heuvel M, and Ingham PW (1996). smoothened encodes a receptor-like serpentine protein required for hedgehog signalling. *Nature* 382, 547–551. [PubMed: 8700230]
- Wang X, Moon J, Dodge ME, Pan X, Zhang L, Hanson JM, Tuladhar R, Ma Z, Shi H, Williams NS, et al. (2013). The development of highly potent inhibitors for porcupine. *Journal of medicinal chemistry* 56, 2700–2704. [PubMed: 23477365]
- Waterhouse AM, Procter JB, Martin DM, Clamp M, and Barton GJ (2009). Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25, 1189–1191. [PubMed: 19151095]
- Woods IG, and Talbot WS (2005). The you gene encodes an EGF-CUB protein essential for Hedgehog signaling in zebrafish. *PLoS biology* 3, e66. [PubMed: 15660164]

Zhang Y, Bulkley DP, Xin Y, Roberts KJ, Asarnow DE, Sharma A, Myers BR, Cho W, Cheng Y, and Beachy PA (2018). Structural Basis for Cholesterol Transport-like Activity of the Hedgehog Receptor Patched. *Cell* 175, 1352–1364 e1314. [PubMed: 30415841]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- The Hedgehog pathway RND proteins DISP1 and PTCH1 are cation-powered transporters
- DISP1 uses the transmembrane Na^+ gradient to catalyze cholesterylated SHH secretion
- PTCH1 uses the transmembrane K^+ gradient to block SMO activation by cholesterol
- Key residue conservation suggests how ion fluxes couple to substrate transport

Figure 1.

Reconstitution of DISP1-dependent SHH release in cultured cells

(A) Schematic of the Hh signaling pathway, showing the two RND-related proteins, DISP1 and PTCH1, which play essential roles in SHH ligand release and in signal transduction, respectively.

(B) DISP1 is required for release of SHH as a soluble complex with a SCUBE family protein. Whether DISP1 acts as a catalytic transporter of cholesterylated SHH, and how DISP1 is powered, are open questions.

(C) Wild-type or *DISP1* knockout (*DISP1*^{KO}) HEK293T cells were co-transfected with SHH, HA-tagged SCUBE2, and the indicated EGFP-tagged DISP1 constructs. SHH release into serum-free media was assayed by immunoblotting. SHH is not released in the absence of DISP1, which is reversed by wild-type DISP1, but not by the inactive DISP1-NNN mutant. Arrowheads indicate the band corresponding to dually lipid-modified SHH.

(D) Schematic of the SHH-NL5 fusion (PDB ID: 6e1h.c and 5ibo).

(E) SHH-NL5 or SHH was stably expressed in HEK293T cells, and release by purified SCUBE2 (500nM) was measured by anti-SHH immunoblotting and densitometry. Released amounts were normalized to the level of SHH species in each cell line. SHH-NL5 and untagged SHH are released with similar kinetics. See also Fig.S1G–J for blots and luminescence measurements.

(F) SHH-NL5 was stably expressed in wild-type or *DISP1*^{KO} HEK293T cells, and SHH-NL5 release by purified SCUBE2 (300nM) into serum-free media was measured by luminescence. SHH-NL5 release by bovine serum albumin (BSA, 300nM) served as

negative control. Defective SHH-NL5 release in $DISP1^{KO}$ cells is rescued by expression of wild-type DISP1, but not by the inactive DISP1-NNN mutant. Data represent mean of two biological replicates and error bars show standard deviation around the mean. See also Figure S1 for characterization of SHH-NL fusions and for recombinant SCUBE2 purification.

Figure 2.

DISP1 catalyzes SHH release from cells

(A) Schematic of the release assay. Cells stably expressing SHH-NL5 are washed of serum, and protein synthesis is inhibited by pre-incubation with cycloheximide (CHX). SHH-NL5 release is initiated by addition of purified SCUBE2, and SHH-NL5 in the media is measured at various time points, by NanoLuc luminescence. Bovine serum albumin (BSA) and methyl- β -cyclodextrin (M β CD) serve as controls.

(B) Kinetics of SHH-NL5 release by a fixed concentration of purified SCUBE2 (1 μ M). SCUBE2 strongly stimulates SHH-NL5 release, while BSA (1 μ M) has no effect. SHH-NL5 release by SCUBE2 is linear over the 30-minute duration of the experiment. Inset shows SHH-NL5 release can be robustly detected 1 minute after addition of SCUBE2.

(C) SHH-NL5 initial release rates. Data represent slope estimates in (B) with error bars showing standard error of the estimate.

(D) Time course of SHH-NL5 release by different concentrations of purified SCUBE2.

(E) SHH-NL5 initial release rates from the experiment in (D), plotted as function of SCUBE2 concentration. Data represent slope estimates and error bars standard error of the estimate. Data were fit to the Michaelis–Menten equation using least-squares, non-linear regression.

(F) As in (E), but with SHH-NL5 stably expressed in wild-type cells, DISP1^{KO} cells, or cells overexpressing wild-type DISP1. Initial release rates were normalized to SHH-NL5 expression levels in each cell line, and to total cell numbers (total protein) in each experimental condition. SHH-NL5 initial release rates increase with increasing DISP1 levels, while displaying saturation relative to SCUBE2. Curves show fits either to the

Michaelis–Menten equation, or to a line in the case of $DISP1^{KO}$ cells. Vertical dotted line shows K_M estimate. See also Fig.S2D, E.

(G) Estimates of V_{max} for the indicated conditions in (F). Error bars indicate standard error of the estimate.

(H) Estimates of K_M for the indicated conditions in (F). Error bars indicate standard error of the estimate. ns. – not statistically significant

(I) as in (F), but with SHH-NL5, $DISP1$ -expressing cells sorted into low- or high-expressers. See also Fig. S2F, G.

(K) as in (G), but with data from (I).

(J) as in (H), but with data from (I).

(L) SCUBE2-dependent SHH-NL5 release as a function of increasing SHH-NL5 levels. Variable amounts of SHH-NL5 were expressed in cells with endogenous $DISP1$ and initial release rates recorded for low ($1\mu M$) and high ($10\mu M$) SCUBE2. At high SCUBE2 concentrations, release saturates as a function of SHH-NL5 level, as indicated by good hyperbolic fit ($R^2=0.998$). Inset shows Lineweaver–Burk plot of the data with accompanying linear fits ($R^2=0.996$ and $R^2=0.995$ for low and high SCUBE2 respectively). Data represent rate estimates versus mean SHH-NL5 level. Vertical error bars represent standard error of the estimate; horizontal error bars represent standard deviation of the mean. See also Fig.S2O.

See also Figure S2 for additional characterization of SHH-NL5 release.

Figure 3.

The Na^+ gradient drives DISP1-catalyzed SHH release

(A) Schematic of Na^+ and K^+ gradients at the plasma membrane in resting mammalian cells.

(B) Cells stably expressing SHH-NL5 were incubated with SCUBE2 (1 μM), in normal Tyrode's media (4KT), 4KT supplemented with gramicidin (2 $\mu\text{g}/\text{mL}$), or media with high K^+ and low Na^+ (Tyrode's containing 125 mM KCl, 125KT), and SHH-NL5 release was measured. Protein synthesis was inhibited with CHX (100 $\mu\text{g}/\text{mL}$). Dissipating Na^+ and K^+ gradients with gramicidin or 125KT inhibits SHH-NL5 release. Data represent rate estimates with error bars showing the standard error of each estimate.

(C) As in (B), but with incubation in Tyrode's media in which Na^+ is replaced with K^+ , Cs^+ , or choline $^+$. Replacement of extracellular Na^+ inhibits SHH-NL5 release in a dose-dependent manner, indicating that the Na^+ gradient is required for DISP1 activity. Electrochemical driving force (EDF) was calculated as described in STAR Methods. Data represent rate estimates; error bars are small and obscured, but indicate the standard error of each estimate.

(D) Decreasing extracellular Na^+ concentration causes rapid and reversible inhibition of SHH-NL5 release. SCUBE2-dependent SHH-NL5 release was measured as in (B), from cells successively incubated in media with physiological Na^+ (4KT), then with low Na^+ and high choline $^+$ (140ChoT), and finally with physiological Na^+ again (Well B). In parallel, cells were incubated successively only in media with physiological Na^+ (Well A), or only in

media with low extracellular Na^+ (Well C). Data represent rate estimates with error bars showing the standard error of each estimate.

(E) As in (B), but with incubation in media with varying Na^+ concentration. Equal osmolarity was maintained with addition of either Cs^+ or choline $^+$. SHH-NL5 release is accelerated by a steeper than normal Na^+ gradient. Data represent rate estimates; error bars are small and obscured, but indicate the standard error of each estimate. Curves show linear fits with associated R^2 values. Vertical dotted lines indicate the physiological value of EDFNa^+ .

(F) As in (E), but using SHH-NL5-expressing DISP1^{KO} cells. SHH-NL5 release is not accelerated by a steeper Na^+ gradient in the absence of DISP1.

(G) As in (E), but using M β CD (1mM) or BSA (5 μ M) instead of SCUBE2. SHH-NL5 release is not accelerated by a steeper Na^+ gradient in the absence of SCUBE2.

(H) As in (E), but with L cell stably expressing WNT3A-NL, and measuring WNT3A-NL release into media containing 2% FBS. WNT3A-NL release is unaffected by changes in the Na^+ gradient. WNT3A-NL faithfully mimics serum- and palmitoylation-dependent release of untagged WNT3A (see Fig.S3K, L).

(I) As in (E), but with incubation in media with varying K^+ concentration, and constant Na^+ gradient. The rate of SHH-NL5 release is unaffected by changes in the K^+ gradient.

See also Figure S3 for additional experiments demonstrating the role of the Na^+ gradient and for additional controls.

Figure 4.

The Na⁺ gradient is dispensable for PTCH1 function

(A) PTCH1 is required for repressing SMO in Hh-responding cells, by antagonizing SMO activation by cholesterol. It is unknown what powers the essential activity of PTCH1.

(B) Confluent NIH-3T3 cells were serum-starved overnight, to promote ciliogenesis, and were then incubated for 3 hours in normal Tyrode's media (4KT) or in media in which Na⁺ was replaced by choline⁺ (140CholT). Ciliary levels of endogenous SMO were measured by immunofluorescence microscopy. Staining for the cilium-resident protein ARL13B was used to identify cilia. Abolishing the Na⁺ gradient does not cause SMO ciliary accumulation. The SMO agonist SAG (500nM) causes ciliary accumulation of SMO in the absence of the Na⁺ gradient. Data show mean of three biological replicates, and error bars show standard deviation around the mean. At least 100 cilia were recorded per replicate.

(C) As in (B), but varying the concentration of choline⁺ replacing extracellular Na⁺. Varying the Na⁺ gradient does not lead to SMO ciliary accumulation. SMO responds to SAG irrespective of Na⁺ concentration. Data show mean of three biological replicates, and error bars show standard deviation around the mean. At least 100 cilia were recorded per replicate.

(D) As in (B), but measuring fluorescence intensity of endogenous GLI proteins in cilia.

Low Na⁺ does not lead to GLI accumulation in cilia, in contrast to SAG or high extracellular K⁺ (110KT, see below). At least 300 cilia per condition were measured in a single biological replicate. * p<0.05, unpaired, two-tailed t-test.

(E) As in (B), but replacing extracellular Na⁺ with NMDG⁺ (110NMDG-T) or K⁺. SMO is not recruited to cilia in 110NMDG-T, in contrast to SAG or 110KT.

Figure 5.

The K^+ gradient is necessary for PTCH1 function

(A) Ciliated NIH-3T3 cells were incubated for 3 hours in media with either low (4KT, 4RbT) or high (110KT, 110RbT) K^+ or Rb^+ , and endogenous SMO at cilia was measured by immunofluorescence. High K^+ or Rb^+ lead to SMO accumulation in cilia, which is reversed by the SMO antagonist, SANT-1 (1 μ M). SAG (500nM) was used as positive control. Data represent mean of at least two biological replicates; error bars show standard deviation around the mean. At least 100 cilia were assayed per replicate. * $p < 0.05$, unpaired, two-tailed t-test.

(B) As in (A), but with incubation in isotonic media in which Na^+ is replaced with various concentrations of monovalent cations (Li^+ , K^+ , Rb^+ , Cs^+). Hill equation curves with Hill slope of 3 and maximum value of 100% were fit to the data. Data represent mean of at two biological replicates; error bars show standard deviation around the mean. At least 100 cilia were assayed per replicate.

(C) As in (A), but measuring endogenous GLI proteins in cilia. GLI proteins are recruited to cilia in media with high extracellular K^+ (110KT), which is reversed by SANT-1. The Hh pathway activators SHH and SAG were used as positive controls. At least 250 cilia were assayed per treatment condition in a single biological replicate. * $p < 0.001$, one-way ANOVA.

(D) As in (A), but measuring transcription of *Gli1* by quantitative RT-PCR, after incubation for 3 and 6 hours. *Gli1* transcription is activated in 110KT media, which is reversed by SANT-1. SHH was used as positive control.

(E) Reducing the K^+ gradient causes reversible inhibition of PTCH1. NIH-3T3 cells were stimulated overnight with SHH, after which they were chased in media of varying K^+ concentration, and ciliary SMO was measured at different times. Ciliary SMO levels drop quickly in 4KT media, but persist in 110KT, in which PTCH1 is inactive. Switching cells from 4KT to 110KT inhibits PTCH1 and thus SMO removal from cilia, which is reactivated

by switching back to 4KT media. At least 250 cilia were assayed per condition in a single biological replicate.

(F) Ciliated NIH-3T3 cells stably expressing EGFP-tagged PTCH1 were incubated for 3 hours in the indicated media, in the presence or absence of SHH, and PTCH1 levels in cilia were measured by immunofluorescence. High levels of extracellular K^+ , Rb^+ , or Cs^+ do not perturb SHH-induced removal of PTCH1 from cilia. PTCH1 fluorescence intensity at cilia is shown as box-and-whisker plots for a single biological replicate. The dotted line shows the threshold used to compute the percent PTCH1-positive cilia (Fig.S4D). At least 100 cilia were assayed per condition.

See also Figure S4 for additional experiments demonstrating the role of the K^+ gradient in Hh signaling

Figure 6.

A conserved cation-sensitive network necessary for DISP1 and PTCH1 function

(A) Sequence alignment of DISP1, PTCH1, and *E. coli* AcrB and AcrD. TM helices are shown as yellow boxes, with red dots indicating polar residues necessary for DISP1, PTCH1 and AcrB function.

(B) Proton-dependent conformational changes in AcrB (PDB ID: 2gif). TM4-resident D407 and D408 residues alternate between being deprotonated and bound to the TM10-resident K940 (left), and being protonated and with K940 bound to T978/S979 in TM11 (right).

(C) Proposed model of cation-dependent conformational switching in DISP1 and PTCH1.

TM10 hosts an aspartate in DISP1 and glutamate in PTCH1, instead of a lysine. This suggests that the deprotonated acidic residues in TM4 and TM10 form a cation-binding site.

In the absence of cation, the first TM4 aspartate binds to the S/T cluster in TM11, to effect conformational change. Residue numbers are for mouse DISP1 and PTCH1.

(D) TM-resident polar residues in DISP1 are necessary for SHH release. DISP1^{KO} HEK293T cells stably expressing SHH-NL5 were stably rescued with DISP1 mutants, and SCUBE2-dependent SHH-NL5 release was measured. Protein synthesis was inhibited with CHX (100µg/mL). Data show estimate of release rate normalized to DISP1 and SHH-NL5 levels in single biological replicate; error bars represent combined standard error and standard deviation around the mean.

(E) TM-resident polar residues in PTCH1 are necessary for SMO repression. PTCH1 mutants were stably expressed in *Ptch1*^{-/-} MEFs, and their ability to reduce ciliary levels of endogenous SMO was assayed by immunofluorescence. SAG (500nM) and SANT-1 (1µM) served as positive and negative control, respectively. Error bars represent standard deviation around the mean of two biological replicates. At least 100 cilia were assayed per replicate. * p<0.01, unpaired, two-tailed t-test.

(F) As in (D), but HEK293T cells stably expressing SHH-NL5 were incubated with SCUBE2 (1µM), in modified Tyrode's media with high Na⁺, choline⁺ or Cs⁺, buffered to different pH values. SHH-NL5 release is potentiated by increasing pH, but only if the Na⁺ gradient is present. Error bars are obscured, but indicate standard error of the estimate in single replicate.

See also Figure S5 for evidence of an alternating hydrogen-bonding network in PTCH1, similar to AcrB. See also Figure S6 for characterization of cells used for DISP1 and PTCH1 mutant analysis.

Figure 7.

RND-catalyzed cholesterol transport in the Hh pathway

(A) Schematic of the prototypical prokaryotic H⁺-powered RND transporter, *E. coli* AcrB.

(B) DISP1 functions as a Na⁺-powered antiporter, transporting cholesterylated SHH from the outer leaflet of the plasma membrane and handing it off to extracellular SCUBE2.

(C, D) Two possible modes of K⁺-powered cholesterol transport by PTCH1. In (C), PTCH1 moves cholesterol between the two leaflets of the membrane, making it unavailable for SMO activation. In (D), PTCH1 transports cholesterol from SMO to the membrane, thus catalyzing SMO deactivation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
rabbit monoclonal anti-SHH (clone C9C5)	Cell Signaling Technology	Cat# 2207S; RRID: AB_2188191
rabbit monoclonal anti-WNT3A (clone C64F2)	Cell Signaling Technology	Cat# 2721; RRID: AB_2215411
rabbit polyclonal anti-GFP	Rockland	Cat# 600-401-215; RRID: AB_828167
mouse monoclonal anti-tubulin (clone DM1A)	Sigma; Blose et al., 1984	Cat# T6199; RRID: AB_477583
mouse monoclonal anti-acetylated tubulin (clone 6-11B-1)	Sigma	Cat# T7451; RRID: AB_609894
goat polyclonal anti-mSMO	Nedelcu et al., 2013	N/A
rabbit polyclonal anti-mCherry	Nedelcu et al., 2013	N/A
chicken polyclonal anti-mARL13B	This study	N/A
rat anti-HA (clone 3F10)–HRP conjugate	Roche	Cat# 12013819001; RRID: AB_390917
sheep anti-mouse IgG–HRP conjugate	Jackson ImmunoResearch	Cat# 515-005-003; RRID: AB_2340287
donkey anti-rabbit IgG–HRP conjugate	GE Healthcare	Cat# NA934; RRID: AB_772206
donkey anti-goat IgG–Alexa Fluor 488 conjugate	Thermo	Cat# A-11055; RRID: AB_2534102
donkey anti-rabbit IgG–Alexa Fluor 555 conjugate	Thermo	Cat# A-31572; RRID: AB_162543
donkey anti-chicken IgY–Alexa Fluor 647 conjugate	Jackson ImmunoResearch	Cat# 703-605-155; RRID: AB_2340379
Bacterial and Virus Strains		
<i>E. coli</i> BL21 (DE3) pLysS	Sigma	Cat# 69451
Chemicals, Peptides, and Recombinant Proteins		
polyethylenimine (PEI), linear, MW 25000	Polysciences	Cat# 23966
TransIT®-293 Transfection Reagent	Mirus	Cat# MIR2700
cycloheximide (94%)	Sigma	Cat# C7698
methyl- β -cyclodextrin (M β CD)	Sigma	Cat# 332615
IWP-2 (PORCN inhibitor) (98%)	Sigma; Wang et al., 2013	Cat# I0536
SAG (SMO agonist) (98%)	Axxora; Chen et al., 2002	Cat# BV-1939
SANT-1 (SMO antagonist) (95%)	Calbiochem; Chen et al., 2002	Cat# 559303
gramicidin A	Sigma	Cat# 50845
veratridine (98%)	Tocris Bioscience	Cat# 2918
bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC ₄ (3)) (95%)	Sigma	Cat# D8189
carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (97%)	Sigma	Cat# C2759
staurosporine (>99%)	LC Laboratories	Cat# S-9300
oligomycin A (99%)	Sigma	Cat# 75351
See Table S1 for composition of modified Tyrode's media utilized in this study.	This study	N/A
FLAG peptide: NH ₂ -DYKDDDDK-OH	Genscript	N/A
6xHis-mARL13B (antigen for immunization)	This study	N/A
MBP-mARL13B (for antibody affinity purification)	This study	N/A
FLAG-HT7-mSCUBE2- Furin	This study	N/A

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical Commercial Assays		
MiSeq Reagent Kit v2	Illumina	Cat# MS-102-2001
Affi-Gel 10 Gel	Bio-Rad	Cat# 1536099
anti-FLAG M1 resin	Millipore	Cat# A4596
Amicon Ultra-4 Centrifugal Filter Unit – 10kDa cutoff	Millipore	Cat# UFC8010
Superdex 200, 10/300 GL	GE Healthcare	Cat# 17517501
Nano-Glo® Luciferase Assay	Promega	Cat# N1120
BCA Assay	G Biosciences	Cat# 786-570
TRIzol Reagent	Thermo	Cat# 15596018
RQ1 RNase-Free DNase	Promega	Cat# M6101
LunaScript RT SuperMix Kit	NEB	Cat# E3010L
PowerUp SYBR Green Master Mix	Thermo	Cat# A25742
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	Cat# G7572
MTT Cell Proliferation Assay Kit	Cayman Chemical	Cat# 10009365
Experimental Models: Cell Lines		
Human: HEK293T	ATCC	Cat# CRL-3216; RRID: CVCL_0063
Human: HEK293S GnTI ⁻	A.C. Kruse; ATCC; Reeves et al., 2002	Cat# CRL-3022; RRID: CVCL_A785
Mouse: L cell	ATCC	Cat# CRL-2648; RRID: CVCL_4536
Mouse: L Wnt-3A	ATCC	Cat# CRL-2647; RRID: CVCL_0635
Mouse: NIH-3T3	ATCC	Cat# CRL-1658; RRID: CVCL_0594
Mouse: <i>Ptch1</i> ^{-/-} MEF	Goodrich et al., 1997	N/A
Human: <i>DISP</i> ^{KO} HEK293T	This study	N/A
Oligonucleotides		
m <i>Rpl27</i> qRT-PCR forward primer: 5'-GTCGAGATGGGCAAGTTCAT-3'	Nedelcu et al., 2013	N/A
m <i>Rpl27</i> qRT-PCR reverse primer: 5'-GCTTGGCGATCTTCTTCTTG-3'	Nedelcu et al., 2013	N/A
m <i>Gli1</i> qRT-PCR forward primer: 5'-GGCCAATCACAAGTCAAGGT-3'	Nedelcu et al., 2013	N/A
m <i>Gli1</i> qRT-PCR reverse primer: 5'-TTCAGGAGGAGGGTACAACG-3'	Nedelcu et al., 2013	N/A
See Table S2 for a list of CRISPR gRNA oligonucleotides and sequencing primers utilized in this study.	This study	N/A
Recombinant DNA		
See Table S3 for a list of all plasmids utilized in this study.	This study	N/A
Software and Algorithms		
MetaMorph Microscopy Automation and Image Analysis Software	Molecular Devices	http://www.moleculardevices.com/Products/Software/Meta-Imaging-Series/MetaMorph.html ; RRID: SCR_002368
MATLAB	MathWorks	http://www.mathworks.com/products/matlab/ ; RRID: SCR_001622
FIJI	National Institutes of Health	http://fiji.sc ; RRID: SCR_002285

REAGENT or RESOURCE	SOURCE	IDENTIFIER
JalView	Waterhouse et al., 2009	http://www.jalview.org/ ; RRID: SCR_006459
PyMOL	Schrodinger	http://www.pymol.org/ ; RRID:SCR_000305
Prism 8	GraphPad	http://www.graphpad.com/ ; RRID: SCR_002798
Photoshop CS5	Adobe	https://www.adobe.com/products/photoshop.html ; RRID: SCR_014199
Inkscape	Inkscape	https://inkscape.org/en/ ; RRID: SCR_014479