Direct visualization of cAMP signaling in primary cilia reveals up-regulation of ciliary GPCR activity following Hedgehog activation

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The primary cilium permits compartmentalization of specific signaling pathways, including elements of the Hedgehog (Hh) pathway. Hh transcriptional activity is thought to be negatively regulated by constitutively high ciliary cAMP maintained by the $G\alpha(s)$ -coupled GPCR, GPR161. However, cilia also sequester many other $G\alpha(s)$ -coupled GPCRs with unknown potential to regulate Hh. Here we used biosensors optimized for ciliary cAMP and strategies to isolate signals in the cilium from the cell body and neighboring cells. We found that ciliary cAMP was not elevated relative to cellular cAMP, inconsistent with constitutive cAMP production. $G\alpha(s)$ -coupled GPCRs (e.g., the 5-HT₆ serotonin and D1R dopamine receptor) had reduced ability to generate cAMP upon trafficking to the ciliary membrane. However, activation of the Hh pathway restored or amplified GPCR function to permit cAMP elevation selectively in the cilium. Hh therefore enables its own local GPCR-dependent cAMP regulatory circuit. Considering that GPCRs comprise much of the druggable genome, these data suggest alternative strategies to modify Hh signaling.

FRET biosensors | calcium | cyclic AMP | primary cilium

Compartmentalization of signal transduction is a recurring theme throughout biological systems. Localized signaling promotes energetic efficiency, maintains fidelity, and allows a limited repertoire of second messengers (e.g., cAMP and Ca²⁺) to be repurposed to control multiple (sometimes conflicting) biological outcomes (1). The primary cilium is an organelle that has been described as a signaling hub because it concentrates a large number of specialized signaling components within its tiny volume. In particular, primary cilia host diverse GPCRs connected to cAMP signaling (2, 3). Ciliary GPCRs linked to $G\alpha_s$ to catalyze the formation of cAMP include 5-HT₆, V2R, EP4, D1R, and TGR5, while SSTR3, NPY2R, KISS1R, MC4R, and MCHR1 are among the ciliary receptors that inhibit adenylyl cyclase through $G\alpha_i$ (2, 4, 5). This ciliary localization is sometimes quite exclusive. For example, the 5-HT₆ receptor, a neuronal GPCR with apparent functions in cognition and memory consolidation (6), can be highly restricted to the cilium (7–9). $G\alpha_s$ (GNAS), adenylyl cyclases (ADCY3, 5, 6, and 10), phosphodiesterases (PDE4C), PKA holoenzyme, and numerous PKA substrates are also established residents of the primary cilium (10, 11); (12–16). Taken together, an extremely attractive model can be envisaged in which 2 cAMP signaling compartments are maintained concurrently in cilium and cell, allowing the same soluble messenger (cAMP) to independently control distinct biological functions. However, the nature of the ciliary signaling microdomain and direct proof that GPCRs function in their usual way have been little explored.

Interest in ciliary cAMP signaling has expanded greatly following the discovery that cAMP-dependent protein kinase A (PKA) is among the kinases regulating the ciliary transcription factors that mediate canonical Hedgehog (Hh) signaling (i.e., through phosphorylation of Gli transcription factors) (17, 18). The prevailing view is that a high resting [cAMP]_{cilia} (relative to the cytosol) is maintained in the absence of Hh ligand, leading to PKA-dependent modulation of Gli (15, 17, 19). GPR161, an orphan receptor that

exits the cilium upon Hh activation, is believed to be constitutively active and has been proposed to supply the cAMP needed for suppression of Hh signaling under resting conditions (17, 18). Meanwhile, smoothened (Smo), a GPCR-like protein central to activation of the Hh signaling, enters the cilium following Hh activation (20). Smo is thought to work through $G\alpha_i$ to decrease cAMP signaling in the cilium and is therefore permissive for Gli engagement.

From this model, it is apparent that additional ciliary GPCRs might impinge on Gli proteins and other ciliary targets. However, the cAMP signaling properties of the cilium have mostly been inferred, e.g., from cAMP measured in whole cells (17, 21–23), and in only a few cases has the second messenger been measured directly in this organelle (19, 24, 25). Applying in vitro calibration curves to fluorescence levels measured from a cilium-targeted cAMP reporter, Moore et al. (19) estimated resting [cAMP]_{cilia} in mIMCD3 cells to be ~4.5 μ M, compared with <1 μ M in the cytosol. This [cAMP] would be fully able to persistently activate ciliary cAMP effectors such as PKA and Epac and thus maintain repression of Hh.

Here we revisited the question of whether GPCR-dependent cAMP signals can be generated independently within the cilium. Using Förster resonance energy transfer (FRET)-based reporters optimized for [cAMP]_{cilia} measurements, we focused on 5 GPCRs previously reported to reside in cilia, EP4, V2R, D1R, SSTR3, and 5-HT₆. The cAMP-generating activity of certain GPCRs was significantly up-regulated by Hh signaling. In conflict with prior reports (19), we also did not find evidence for elevated basal [cAMP] in the cilioplasm of 3 different cell types. Our direct organelle measurements challenge some of the assumptions about the nature of cAMP signaling in the ciliary space and indicate possible roles for GPCRs other than GPR161 in Hh repression.

Significance

The primary cilium, a small organelle essential to normal development, has recently been thrust into the limelight owing to its involvement in adult disease states such as cancer, obesity, neurodegeneration, and polycystic kidney disease. Here we developed optical approaches to interrogate the function of receptors that work through cAMP-PKA signaling within the primary cilium of live cells. Our data provide a key piece of the puzzle concerning the way in which localized cAMP signals regulate the Hedgehog pathway, another ciliary signaling system with emerging roles in multiple disease processes.

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Fig. 2. Resting ciliary cAMP is not elevated relative to the cytosol. (A) Ciliary cAMP was calibrated using loosely targeted Arl13b-Epac-H188 (K_d for cAMP ~ 10 μ M) in digitonin-permeabilized mIMCD3 cells and serially increasing cAMP buffer concentrations. The resulting response curves were not significantly different in cilia versus cell body (typical of $n = 4$ experiments, 7 primary cilia/cells). (B) Calibration of Arl13b-Epac-H187 was performed as above ($n = 6$ experiments with 6 primary cilia/cells, $P = 0.9741$). See also *SI Appendix, Fig. S3* for additional details. Blue traces, cell body; red traces, cilia. Time bar: 3 min. The y axis depicts the 480/535 FRET ratio.

of ciliary cAMP. Assuming that the endogenous EP4 receptor is localized to cilia in our cells, this signal could be derived either from within the cilium through activation of ciliary EP4 receptors, via communication with cAMP synthesized in the cell body, or with some combination thereof.

The V2R vasopressin receptor was reported to reside on the cilium of renal cells (32). As mIMCD3 cells proved to have no endogenous expression of functional V2R, we transfected cells with mRuby2-, mScarlet-, or EGFP-tagged versions of the receptor. Analysis of the 3-dimensional distribution of V2R in these cells showed that the vast majority of the GPCR was in the plasma membrane, with a relatively low density of V2R in the cilium (Fig. 3D, arrow; see *SI Appendix, Fig. S5* for quantification). Expression of these constructs rendered the cells uniformly sensitive to low physiological concentrations of AVP (Fig. 3E). Interestingly, in contrast to PGE1, the response to AVP was only rarely reversible after agonist washout, perhaps reflecting the continued activation of internalized V2R from an endosomal compartment (33, 34). Persistent cAMP elevation was also observed in the primary cilium using targeted sensors; Fig. 3F depicts an experiment in which it was possible to monitor 2 cell bodies and their associated cilia in the same microscope field. The ciliary and cellular signals mirror one another. We conclude that the cAMP signal measured in the cilium after AVP stimulation is generated principally in the cytosol and, as expected (8), communicated freely to the cilium by diffusion.

We then examined the effects of stimulating the ciliary 5-HT₆ receptor. As mIMCD3 cells do not express native 5-HT₆, mCherry-5-HT₆ was coexpressed with targeted or nontargeted cAMP reporters. Fig. 3G illustrates that 5-HT₆ receptors can segregate exclusively to the primary cilium, although cells harboring receptors in both the cilia and cell body were also noted (e.g., see *SI Appendix, Fig. S6*). These latter cells could be reliably stimulated with serotonin (5-HT) to yield increased FRET ratios (Fig. 3H). We expected that we should be able to directly visualize agonist-dependent signals in ciliated cells in which the GPCR was exclusively localized to the cilium. However, to our surprise, we were never able to record a cAMP change in either the cilium or

Fig. 3. Evaluation of putative ciliary GPCRs: EP4, V2R, and 5-HT₆. (A) Three-dimensional Airyscan images of a ciliated mIMCD3 cell expressing EP4-mCherry (red) and Arl13b-GFP (green). (B) FRET ratio changes in multiple mIMCD3 cells stimulated with 100 nM PGE1 as measured by nontargeted Epac-H187, followed by 50 μ M forskolin + 20 μ M rolipram (typical of 4 experiments, 38 cells). (C) Typical ciliary cAMP in response to PGE1 as measured by Arl13b-Epac-H187 (17 experiments, positive responses in 13 of 20 cilia). (D) Three-dimensional Airyscan image of a mIMCD3 cell expressing V2R-mRuby2 (white arrow indicates cilium). (E) Persistent cAMP signal after treatment with 50 pM AVP in mIMCD3 expressing V2R-mRuby2 and cytosolic Epac-H187 (typical of 4 experiments, 38 cells). (F) AVP produced similar cAMP responses in cilia and cell body as measured by Arl13b-Epac-H187 (typical of 6 experiments, 14 cilia). (G) Three-dimensional Airyscan images of a ciliated cell expressing 5-HT₆-mCherry (red) and Arl13b-Epac-H187 (green). (H) mIMCD3 cells expressing poorly targeted 5-HT₆ and nontargeted Epac-H187 responded robustly to 10 μ M 5-HT in ciliated and nonciliated cells (6 experiments, 9/25 cells ciliated). (I) When 5-HT₆ was strictly confined to the ciliary membrane (indicated by mCherry fluorescence), treatment with 5-HT caused no change in cAMP (6 experiments, 6 cilia). In this experiment, there was insufficient FRET sensor in the cytosol to report cytosolic cAMP changes, explaining the lack of response to 50 μ M forskolin and 1 mM IBMX (3-isobutyl-1-methylxanthine: noisy blue dotted trace). (J) When 5-HT₆ was localized to the cell membrane, the inverse agonist, SB742457 (1 μ M), caused a reduction in [cAMP]_{cytosol} levels, and 5-HT reversed the effect (typical of 3 experiments, 16 cells). (K) When 5-HT₆ was localized strictly to the ciliary membrane, SB742457 had no effect on cAMP levels in the cell body or cilia (typical of 7 experiments, 11 cilia). Blue traces, cell body; red traces, cilia. Time bar: 3 min unless otherwise noted; the y axis depicts the 480/535 nm FRET ratio.

cytosol (Fig. 3*I*) under these circumstances. We observed that receptor expression in nonciliated cells results in constitutive elevation of cellular cAMP, as reported previously (6). The constitutive activity of the receptor was unmasked using the inverse agonist SB742457, causing variable reduction in the baseline ratios (Fig. 3*J*), and effectively restored using the native agonist, 5-HT (Fig. 3*J*). Similarly, when 5-HT₆ was perfectly localized, we failed to observe any effect of the inverse agonist on ciliary cAMP (Fig. 3*K*), suggesting that the usual constitutive activity was eliminated once the GPCR was in the ciliary membrane. Note that these experiments were conducted in the presence of gap junction inhibitors to prevent communication of cAMP between ciliated and nonciliated GPCR-expressing cells (35).

We next employed a permeabilized cell model with the aim of isolating the ciliary response from that in the rest of the cell. We initially focused on the 5-HT₆ receptor because we could confirm definitively its ciliary localization. Cells were treated briefly with a pulse of digitonin in an “intracellular-like” buffer supplemented with ATP and GTP and free [Ca²⁺] clamped to 180 nM (Fig. 4*A*). We reasoned that the digitonin would render the cell body incapable of accumulating cAMP following agonist stimulation, while the sensor in the restricted microdomain of the primary cilium should be able to register local cAMP production. A pulse of cAMP was given at the end of every experiment to confirm permeabilization of the plasma membrane. The response to 5-HT in the cell body was completely eliminated following permeabilization. However, again, no response to 5-HT in the cilia was ever observed in mIMCD3 cells under these conditions, even though the organelle clearly harbored the GPCR (Fig. 4*A*). We also saw no effect of the inverse agonist (Fig. 4*B*) under these conditions. Experiments aimed at assessing the activity of EP4 and V2R in permeabilized cells gave comparable results (*SI Appendix*, Fig. S7).

An obvious explanation for the lack of effect in the preceding experiments was that cAMP did not accumulate sufficiently to be captured by the reporter. However, when the same experiment was repeated using forskolin to directly activate endogenous ACs

(adenylyl cyclases) present in the ciliary membrane, a small but consistent accumulation of cAMP was detected in the microenvironment of the cilium but not in the cell body (Fig. 4*C* and *E*, left bar graphs). Similar results were obtained in cells expressing AC3 (36) (Fig. 4*D* and *E*, right bar graphs), which was localized to cilia when expressed in mIMCD3 cells (*SI Appendix*, Fig. S8). Assuming that permeabilization did not lead to loss of essential components of the GPCR signaling machinery, the small but detectable change following forskolin addition suggests that it should have been possible to resolve GPCR-dependent cAMP signals were they to take place in the cilium. These data also indicate that the ciliary membrane of mIMCD3 cells was left intact following digitonin treatment.

The preceding data leave us with the question as to why GPCRs are not competent to produce cAMP once sequestered in the cilium. Prior studies have cited Ca²⁺ entry across the ciliary membrane as an important regulator of organelle cAMP owing to the presence of cilia-specific Ca²⁺-conducting channels (PKD2L1 and PKD2) and Ca²⁺-regulated ACs (AC1, 8, 5, and 6) (11, 16, 23, 32, 37). Primary cilia of mIMCD3 cells have been reported to harbor Ca²⁺-inhibitable AC5 and/or AC6 (10). We therefore considered that the high resting [Ca²⁺] and Ca²⁺ permeability of the cilium (38, 39) could account for the inactivation of ACs coupled to the 5-HT₆ receptor. To better address this point, we improved the sensor by replacing the Arl13b targeting sequence with a functional 5-HT₆ receptor to target the cAMP reporter–GPCR fusion directly to the cilium (5-HT₆-EpacH187). Consistent with the data of Figs. 3*I* and 4*C*, when targeted exclusively to the cilium, 5-HT did not yield a measurable cAMP signal (Fig. 5*A*). Exposure of cells to nominally Ca²⁺ free solutions did not rescue the 5-HT response in cilia (Fig. 5*B*), indicating that Ca²⁺ entry across the ciliary membrane did not suppress the signal. We considered that perhaps expression of 5-HT₆ altered the ciliary distribution of the Gα_s protein in mIMCD3 cells, but immunostaining experiments suggested this is not the case (*SI Appendix*, Fig. S9). However, when we tested the effect of 5-HT in cells pretreated with SAG (250 nM to 1 μM) to activate Smo receptors (40), we were able to restore the responsiveness of the 5-HT₆ receptor to agonist (Fig. 5*C*). Sensitivity to the inverse agonist was also apparent, implying restoration of constitutive activity (Fig. 5*D*).

The preceding results left us with the question of whether the unexpected SAG-dependent up-regulation of the 5-HT₆ receptor expressed in mIMCD3 cells was cell type and/or GPCR specific. Unlike mIMCD3, stimulation of 3T3 cells (Fig. 5*E*) and MEFs (*SI Appendix*, Fig. S10*A*) with 5-HT produced a measurable ratio change that was, however, significantly enhanced following SAG treatment (Fig. 5*F* and *SI Appendix*, Fig. S10*B*). Interestingly, the smoothened receptor antagonist cyclopamine, which might be expected to have the opposite effect of SAG, did not abolish 5-HT-stimulated cAMP signaling in primary cilia of 3T3 cells (bar graph, Fig. 5*F*).

Using this system, we were also able to evaluate the ability of a ciliary Gα_s-coupled GPCR, SSTR3, to reverse serotonin-stimulated cAMP signals in the cilium. Treatment of 3T3 cells coexpressing SSTR3-GFP and 5-HT₆-H187 with somatostatin reversed 5-HT responses back to baseline within 2 min in the presence and absence of SAG treatment (Fig. 5*G* and *H*), indicating local control of the 5-HT₆ function. We then further tested how SAG affected the activity of another ciliary Gα_s-coupled GPCR, the dopamine D1R receptor (5). D1R-EGFP was coexpressed with 5-HT₆-H187 in 3T3 cells, and cells with fluorescence only in cilia were selected. Ten nanomolar dopamine produced small but measurable cAMP signals in control cells (Fig. 5*I*), and again, the signal was significantly enhanced after SAG treatment (Fig. 5*J*; summarized in bar graph).

Discussion

The GPCR/cAMP pathway is considered one of the cornerstones of the “druggable genome.” Thus, the significance of a distinct cAMP signaling circuit in the primary cilium is that it may potentially

Fig. 4. cAMP signaling in cilia of digitonin-permeabilized cells. All experiments were performed in intracellular-like buffer supplemented with ATP and GTP. (A) mIMCD3 cells expressing 5-HT₆-mCherry and Arl13b-H187 localized to both the cell and cilium did not respond to 10 μM 5-HT in the cell body or in the cilium after 2 min treatment with 20 μg/mL digitonin (4 experiments, 5 cilia). (B) Similarly, responsiveness to the inverse agonist, 1 μM SB742457, and subsequent 5-HT₆ rebound response were lost in both the cell and cilium after 2 min treatment with 20 μg/mL digitonin (3 experiments, 3 cilia). (C) Digitonin permeabilization ablated the 50 μM forskolin-induced cAMP signal in cell bodies, but cAMP increase was detectable in cilia; 1 mM IBMX-induced PDE inhibition did not unmask any signal ($n = 4$ experiments with 4 cilia/7 cells). (D) AC3 cotransfection did not change the forskolin response. (E) Quantification and comparison of cAMP response to forskolin in cell bodies and in cilia; $^{**}P = 0.0002$, $^{***}P = 0.00019$. Blue traces = cell body, red traces = cilia. Time bars: 3 min. The y axis is the 480/535 nm ratio.

Fig. 5. Hedgehog pathway activation increases cAMP response reported by 5-HT₆-Epac-H187 in primary cilia: (A) Consistent with the data in Figs. 3I and 4C, mIMCD3 cells did not respond to 10 μM 5-HT when the sensor/receptor fusion was confined strictly to the ciliary membrane (6 experiments, 6 cilia). (B) Attenuating the high [Ca²⁺] in cilia by placing cells in a nominally Ca²⁺ free solution did not rescue 5-HT-stimulated cAMP signaling (5 experiments, 6 cilia). (C) Following overnight pretreatment with 1 μM SAG, 5 out of 6 cilia (6 experiments) responded to 5-HT stimulation (***P* = 0.000992). (D) Pretreatment with 1 μM SAG rescued the response to 1 μM SB742257 (−35.5 ± 11.2% below baseline relative to the subsequent cAMP increase elicited by 10 μM 5-HT; typical of 4 experiments, 4 cilia). (E) A cAMP increase in NIH 3T3 cilia (typical of 9 experiments, 11 cilia) was caused by 10 μM 5-HT. (F) Overnight pretreatment with 1 μM SAG significantly increased 5-HT response (***P* = 0.00034; 6 experiments, 7 cilia). Overnight pretreatment with 10 μM cyclopamine (Cyclo) had a negligible effect (8 experiments, 9 cilia, bar graph). (G and H) The 100 nM somatostatin (SST) attenuated 5-HT response in cilia harboring SST3-GFP with and without SAG pretreatment (G: 5 experiments, 6 cilia; H: 2 experiments, 5 cilia). (I and J) SAG pretreatment increased the 10 nM dopamine (DA) response (I: 4 experiments, 4 cilia; J: 4 experiments, 6 cilia, ***P* = 0.002476). Blue traces, cell body; red traces, cilia. Time bars: 3 min. The y axis is the 480/535 nm FRET ratio. Bar graphs indicate the 5-HT or DA response as a percentage of the forskolin/IBMX response; error bars represent SEM.

be exploited to intervene on other less druggable pathways localized to this organelle (Wnt, PDGFRα, planar cell polarity, etc.). The clearest example to date of a regulatory role for cAMP/PKA in the cilium concerns the Hh pathway (12, 18, 21, 41), aberrations in which are thought to occur in up to 25% of all cancers, including basal cell carcinoma and medulloblastoma (42, 43). Intraciliary cAMP has also been implicated in other processes, such as the elaboration of dendrites (44), ciliary length control (37), and flow sensing in osteocytes (16), and in the etiology of obesity (29, 45). The polycystic kidney disease-related protein PKD2 and other ciliary ion channels are additional potential targets of local cAMP signals

(32, 46), although the identity of these conductances and how they are regulated are only starting to be explored (38, 39); (32, 47–50).

The 5-HT₆ and D1R receptors, with their near-exclusive localization to the cilium and well-developed pharmacology (6, 51), are potentially poised to fulfill the role of selective activators of ciliary cAMP/PKA targets, including Hh. The neuronal 5-HT₆ receptor has attracted much interest due to its apparent positive effect on memory consolidation and cognitive function (6). However, a recent series of clinical trials to test the effectiveness of 5-HT₆ antagonists on Alzheimer's disease yielded disappointing results (52). A more complete appreciation of how and where this GPCR functions could be key to developing more effective drugs.

In this study we overcame some of the technical challenges of measuring dynamic cAMP changes in this small organelle to show that 5-HT₆ receptors, which were fully functional while in the cell body plasma membrane of ciliated and nonciliated mIMCD3 cells, ceased to generate cAMP upon trafficking to the ciliary membrane. Not only did 5-HT fail to elicit the expected elevation of cAMP, but treatment with an inverse agonist revealed that the constitutive activity of the receptor was also abolished under these conditions. Significantly, this behavior was reversed when cells were treated overnight with the SAG, which causes trafficking of Smo to the cilium to initiate Gli-dependent transcription of Hh target genes (40). In other experiments in 3T3 and MEFs, SAG treatment caused significant up-regulation of 5-HT₆ and D1R activity. Our data therefore reveal a condition under which 5-HT₆ and D1R receptors are predicted to become competent to locally produce cAMP, activate PKA, and provide feedback to repress Gli transcriptional activity.

Our experimental protocol utilized gap junction inhibitors to prevent the transfer of cAMP from cells with functional 5-HT₆ receptors in the plasma membrane to neighboring ciliated cells with receptor exclusively localized to the primary cilium. Since our data showed that cAMP readily traverses the cytosol to enter the cilium, cAMP communicated from neighboring cells (35) will also enter the cilium to give the false appearance of a cilium-generated cAMP change. Attention to this detail in future studies may clarify some of the conflicting results in the field.

The question remains of how and why the activity of the GPCRs examined is regulated in the ciliary milieu. We excluded high intraciliary Ca²⁺ (39) as the reason for the lack of effect of 5-HT in mIMCD3 cells (Fig. 5B). It is possible that in the absence of Hh ligand Gα_s-coupled GPCRs in the primary cilium experience alternative coupling to other Gα subunits (e.g., Gα_i). Masyuk et al. (53) provided evidence for such a mechanism using biochemical assays of total cAMP in cholangiocytes expressing the endogenous bile acid receptor, TGR5. Ciliary localization appeared to induce a switch of TGR5 from Gα_s-coupled to Gα_i-coupled activity. Other explanations for the apparent reduction in GPCR activity include the distinct lipid composition of the ciliary membrane (54), which may not support GPCR function, and the absence of appropriate Gβγ subunits in the cilium (55). SAG treatment might alter the expression, activity, or ciliary accumulation of adenylyl cyclases, cAMP phosphodiesterases, heterotrimeric G proteins, or arrestins. It could also affect the recruitment of other unknown GPCRs that antagonize Gα(s)-coupled receptors or lead to changes in the lipid composition of the cilium, ultimately affecting the ability of GPCRs to produce cAMP.

In contrast to prior reports (19), we also did not observe elevated basal [cAMP] in the cilium, nor was baseline ciliary [cAMP] altered by activation of the Hh pathway with SAG or Shh (Fig. 2). These data therefore challenge the assumption that cAMP production occurs constitutively in the ciliary compartment. Our findings do not discount the large body of experimental evidence indicating that ciliary targets are actively regulated by the cAMP/PKA pathway (12, 14, 17, 18, 22, 25). They do, however, indicate that a more complex cAMP signaling landscape must exist than previously proposed. Most significantly, our data show Smo

stimulation fosters the development of alternative pathways to local cAMP production. GPCRs such as 5-HT₆, with its well-developed pharmacology and tight localization to cilia, may be adjuncts to therapies that intersect with aberrant Hh signaling.

Materials and Methods

For full details see *SI Appendix, Materials and Methods*. Epac1-based cAMP sensors (26), "Epac-H187" and "Epac-H188" (a gift from Dr. Kees Jalink, Netherlands Cancer Institute, Amsterdam, The Netherlands), were targeted to cilia by conjugation with Arl13b or 5-HT₆. Real-time FRET imaging experiments were performed on a Nikon Eclipse TE2000-U inverted fluorescence microscope equipped with an ORCA ER camera and a 60× Plan Apo TIRF (N.A. = 1.45) oil immersion objective using 440 nm excitation

and a 480 nm/535 nm emission ratio (35, 56). Resting cAMP in cilia and cell bodies was determined by careful calibration of targeted and untargeted cAMP sensors in digitonin permeabilized cells.

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