Supplementary Table 1. Tests of statistical significance[a] for all figures.

Figure	Condition 1	Condition 2	Significant?	p-value
3a	multiple comparison	multiple comparison	yes	<0.001 [b]
3c	Ctrl	Wt	yes	<0.0001 [c]
3c	WRR	Wt	yes	<0.0001 [c]
3c	Ctrl	WRR	no	n.s. [c]
4a	SMO657 wt: βarrestin1	SMO657 wt: NbSmo2	yes	0.00053
4a	SMO657 wt: βarrestin1	SMO657 wt: PKA-C	yes	0.00438
4a	SMO657 wt: PKA-C	SMO657 WRR: PKA-C	yes	0.001553
4a	SMO657 wt: PKA-C	SMO566 wt: PKA-C	yes	0.003181
4a	SMO657 WRR: βarrestin1	SMO657 WRR: PKA-C	no	0.051139
4a	SMO657 WRR: βarrestin1	SMO657 WRR: NbSmo2	yes	0.000165
4a	SMO566 wt: βarrestin1	SMO566 wt: PKA-C	no	0.22268
4a	SMO566 wt: βarrestin1	SMO566 wt: NbSmo2	yes	0.00162
4b	SMO674: wt	SMO674: WRR	yes	<0.0001 [d]
4c	SMO wt: PKA-R	SMO wt: PKA-C	yes	0.012831
4c	SMO wt: PKA-C	SMO WRR: PKA-C	yes	0.01315
4c	SMO WRR: PKA-R	SMO WRR: PKA-C	no	0.113776
5b	SMO: ctrl	SMO: SAG	yes	<0.0001[d]
5b	PKA-C: ctrl	PKA-C: SAG	yes	<0.0001[d]
5b	PKA-C: ctrl	PKA-C: cyc	no	0.2161[d]
6a	None	PKA-C	yes	0.000723
6a	PKA-C	PKA-C + SMO674 wt	yes	0.000995
6a	PKA-C	PKA-C + SMO674 WRR	no	0.267104
6a	PKA-C	PKA-C + SMO674 A635S	no	0.544481
6a	PKA-C + SMO674 wt	PKA-C + SMO674 WRR	yes	0.004314
6a	PKA-C + SMO674 wt	PKA-C + SMO674 A635S	yes	0.000513
6b	Ctrl.: Ctrl	Ctrl.: ShhN	no	0.63116
6b	SMO wt: Ctrl	SMO wt: ShhN	yes	0.010704
6b	SMO wt: ShhN	SMO WRR: ShhN	yes	0.010346
6b	SMO wt: ShhN	SMO A635S: ShhN	yes	0.010945
7b	βarrestin1: SMO WRR	PKA-C (0.04 µg): SMO WRR	no	0.885586
7b	βarrestin1: SMO WRR	PKA-C (0.08 µg): SMO WRR	yes	0.001529
7b	βarrestin1: SMO WRR	PKA-C (0.12 µg): SMO WRR	yes	0.000368
7b	βarrestin1: SMO WRR	PKA-C (0.16 µg): SMO WRR	yes	0.000109
7b	βarrestin1: SMO WRR	PKA-C (0.20 µg): SMO WRR	yes	0.000085
7b	PKA-C (0.04 µg): SMO wt	PKA-C (0.04 µg): SMO WRR	yes	0.004444

7b	PKA-C (0.08 µg): SMO wt	PKA-C (0.08 µg): SMO WRR	yes	0.000502
7b	PKA-C (0.12 µg): SMO wt	PKA-C (0.12 µg): SMO WRR	yes	0.001341
7b	PKA-C (0.16 µg): SMO wt	PKA-C (0.16 µg): SMO WRR	yes	0.004344
7b	PKA-C (0.20 µg): SMO wt	PKA-C (0.20 µg): SMO WRR	yes	0.000029
7b	PKA-C (0.04 µg): SMO566	PKA-C (0.04 µg): SMO WRR	yes	0.00368
7b	PKA-C (0.08 µg): SMO566	PKA-C (0.08 µg): SMO WRR	no	0.180776
7b	PKA-C (0.12 µg): SMO566	PKA-C (0.12 µg): SMO WRR	yes	0.014786
7b	PKA-C (0.16 µg): SMO566	PKA-C (0.16 µg): SMO WRR	yes	0.006063
7b	PKA-C (0.20 µg): SMO566	PKA-C (0.20 µg): SMO WRR	yes	0.001566
ED 6b	SMO wt + PKA-C	SMO WRR + PKA-C	yes	<0.0001[d]
ED 8a	SMO657	SMO657 WRR	no	0.057394
ED 8b	SMO wt	Ctrl	yes	0.003
ED 8b	SMO wt	SMO WRR	no	0.9174
ED 8b	SMO wt	SMO A635S	no	0.4735

[a] All tests of statistical significance were performed as an unpaired Welch's t-test (two-sided) with multiple comparisons corrected using the Holm-Šídák method unless noted otherwise in the table.

[b] To check for significant differences between PKA-C lacking peptide (control) and each peptide concentration a one-way ANOVA with Tukey's multiple comparison was performed. All comparisons show p≤0.0001 (****) significance except the comparison between 125µM standard vs. extended peptide (p≤ 0.001, ***).

[c] To check for significant differences between PKA lacking peptide (control) and the indicated recombinant pCT fragment a one-way ANOVA with Tukey's multiple comparison was performed (**** ≙ p≤0.0001, ns ≙ not significant).

[d]Statistical significance was assessed using an unpaired t-test (two-tailed).

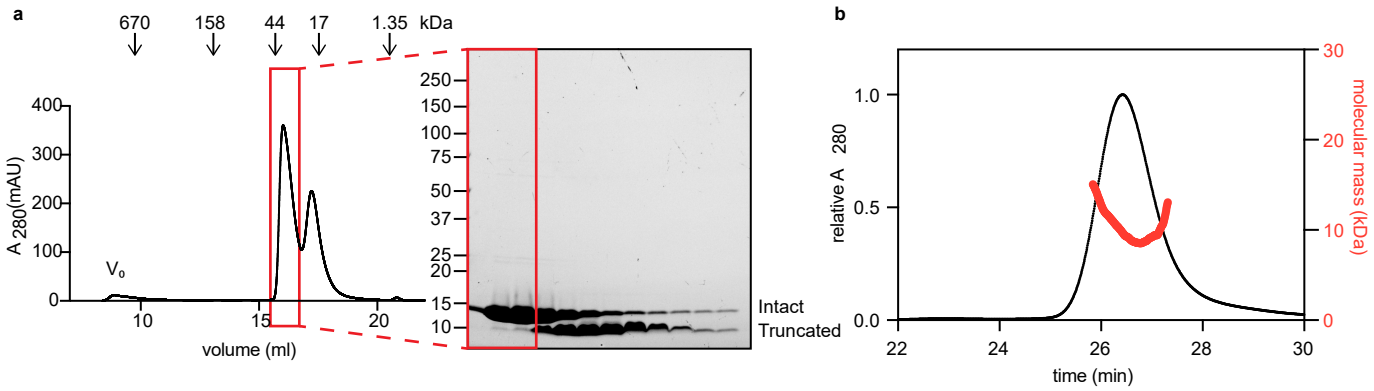
Supplementary Table 2. Quantification of phenotypes for zebrafish embryogenesis studies. The # and % of animals exhibiting U-shaped somites (indicative of a failure in Hh signaling during somitogenesis) are indicated. Note that in both the WRR and A635S mutant conditions, close to 25% of animals exhibited U-shaped somites, consistent with the Mendelian inheritance of a null *smo* allele from the initial heterozygous incross.

mRNA injected	**# Animals from *smo*(+/-) incross**	**# Animals with U-shaped somites**	**% Animals with U-shaped somites**
SMO wt	154	2	1.3%
SMO WRR	226	61	27.0%
SMO A635S	70	19	27.1%

Supplementary Table 3. Sequences of oligonucleotides used in this study.

Oligo Sequence	Oligo description	Used to make
AAGTTCTGTTCCAGGGGCCCGGATCCGACTCAGAAGTCAATCAAGAAG	pHTSHP BamHI SUMO fwd	SMO pCT(565-657) / pHTSHP
CCCACCAATCTGTTCTCTGTGAGCC	SUMO rev	SMO pCT(565-657) / pHTSHP
ACAGAGAACAGATTGGTGGGAAGAGAATCAAGAAGAGCAAGATGA	SUMO-SmoCT fwd	SMO pCT(565-657) / pHTSHP
ATTCAAGCATGCCCGCGGGCGGCCGCtcaGTTGGCTTGTTCTTCTGGTGGCACT	pHTSHP SmoCT-QAN NotI rev	SMO pCT(565-657) / pHTSHP
GCTCAGCATGTCACCAAGATGGTGGCTCGGAGAGGAtCCATATTGCCCCAGGATGTGTCC	mSmo A635S fwd	mSmo A635S constructs
GCTCAGCATGTCACCAAGATGGTGGCTgcGgcAGGAGCCATATTGCCCCAGGATGTGTCC	mSmo R632A R633A fwd	mSmo WRR constructs
AGCCACCATCTTGGTGACATGCTGAGCCgcGGCAGAGGAGACGTCAGCTGAGGGTTCATT	mSmo W622A rev	mSmo WRR constructs
CTGTTCTCCATCAAGAGCAACCACCCTGGGCTCTTAAGTGAGAAGGCAGCCAGCAAGATC	mSmo AflII fwd	mSmo mutant pGEN constructs
TAGGCTCTGGGCAGAAGGGGTTGGA	mSmo PshAI rev	mSmo mutant pGEN constructs
GCTCAGCATGTCACCAAGATGGTGGCT	mSmo A623-A631 fwd	mSmo WRR, A635S constructs
AGCCACCATCTTGGTGACATGCTGAGC	mSmo A623-A631 rev	mSmo WRR, A635S constructs
TGAAACAGAACTTCCAATGCGGCCGCtcaGCCCAAACGCTTCTCTAACTCTGGG	FLAG-SMO(64-674) / pV6 NotI rev	mSmo mutant pVLAD6 constructs
ACCCTGCTCATCTGGAGGCGCACCTGGTGCAGGTTGACTGGGCACAGTGATGATGAGCCC	mSmo DraIII fwd	mSmo mutant pVLAD6 constructs
GAATTCGAAAGAGAATCAAGAAGAGCAAGATGATC	SMO_QAN pGex_Eco_Fw	mSMO pGEX-KG constructs
AAGCTTTCAGTTGGCTTGTTCTTCTGGTG	SMO_QAN_pGex_Hind_rev	mSMO pGEX-KG constructs
AGCTTGGTACCGAGCTCGGATCCACTAGTCCAGTGTGGTGGAATTCTGCAGATATCAACAAGTTTGTACAAAAAAGCAGGCTGCCACCatggcc	Smo-V5-TurboID Forward (5'-3'):	SMO-turboID constructs

GCACAGTCGAGGCTGATCAGCGGGTTTAAACACCACTTTGTACAAGAAAG CTGGGTCTATTAGTCCAGGGT	Smo-V5-TurboID Reverse (5'-3'):	SMO-turboID constructs
CTGTTCTCCATCAAGAGCAACCACCCTGGGCTCTTAAGTGAGAAGGCAGC CAGCAAGATC	mSmo AflII fwd	mSMO wild-type / mutant pCDNA5-FRT-TO constructs
ATGCCCTTCAGCTCGATGCG	GFP HpaI rev	mSMO wild-type / mutant pCDNA5-FRT-TO constructs



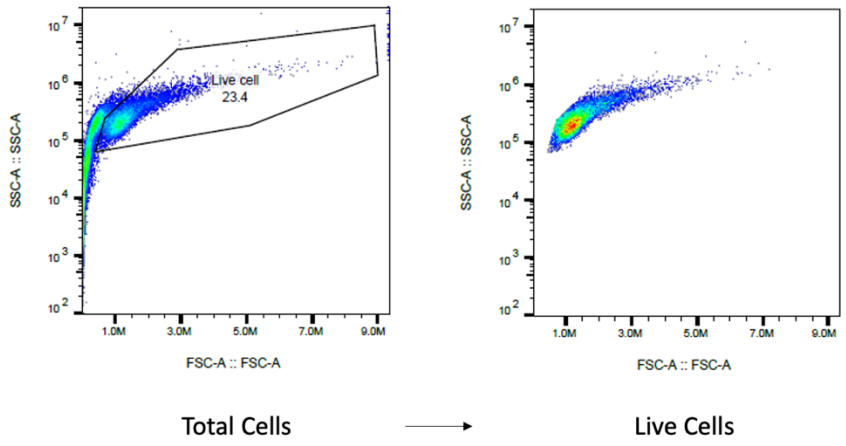
Supplementary Figure 1. Purification of SMO pCT domain from *E. coli.* **a,** Following size exclusion chromatography (left), the purified protein was analyzed by SDS-PAGE (right). The SMO pCT elutes as two peaks, the earlier corresponding to the intact pCT. Fractions containing the intact pCT (red box) were pooled and used for subsequent experiments. Data shown are representative of three independent experiments. **b**, Multi-angle light scattering coupled with size exclusion chromatography (SEC-MALS) was used to determine the protein oligomeric state for the pooled fractions indicated in **a**. The average molecular mass was calculated as M_w = 11.25 +/- 2.1 kDa, close to the predicted molecular mass for a monomer (10.1 kDa).

A PKA Inhibitor Motif within Smoothened Controls Hedgehog Signal Transduction

Supplementary Figure 2

FACS Gating Strategy

(gated on forward scatter (FSC) and side scatter (SSC) only)



Supplementary Figure 2. Gating scheme for flow cytometry studies presented in Extended Data Fig. 8.

Supplementary Discussion.

Supplementary Note 1: SMO / PKA-C colocalization studies in NIH3T3 cells

We found that SMO colocalizes with endogenous PKA-C in NIH3T3 cilia (Fig. 5). We note that our NIH3T3 cells were modified to stably overexpress epitope-tagged SMO, which likely aided in visualizing ciliary PKA-C by boosting the level of its key interacting partner (SMO) in the ciliary membrane. However, our findings are unlikely to be artifacts of SMO overexpression, for two reasons. First, we took great care to construct a stable cell line expressing SMO at very low levels (see "Methods"). We also confirmed that the overexpressed SMO in this clone undergoes physiological regulation by the ciliary trafficking machinery (Extended Data Fig. 7), similar to previous observations with endogenous SMO[1-3]; in contrast, SMO overexpression at high (or even moderate) levels often leads to substantial SMO accumulation in cilia in the Hh pathway "off" state with no further increase in the Hh pathway "on" state[4,5]. Second, SMO / PKA-C colocalization depends strictly on SMO activity state: it occurs only when SMO localizes to cilia in an active (SAG-bound) conformation, and not when SMO localizes to cilia in an inactive (cyclopamine-bound) conformation (Fig. 5). This result rules out the possibility that colocalization is an artifactual consequence of high-level SMO accumulation in the cilium. Instead, our findings are consistent with the view that SMO interacts with a small pool of active (i.e., non-PKA-R-bound) PKA-C critical for Hh signal transduction, reducing its enzymatic activity to low enough levels that GLI phosphorylation is inhibited.

Supplementary Note 2: Assumptions regarding SMO / PKA-C stoichiometry in cilia

The stoichiometry of SMO relative to PKA-C in cilia is likely a critical determinant of Hh pathway activation. Unfortunately, empirical determination of SMO and PKA-C concentrations within the cilium is extremely challenging with currently available biochemical tools. Nevertheless, existing data support the view that levels of SMO in the cilium can exceed those of PKA-C.

First, a recent ciliary proteomic study readily detected endogenous SMO and PKA-R subunits in cilia, but not PKA-C[6]. Given that a pool of active PKA-C critical for Hh signal transduction is known to exist within the cilium[7-10], the most likely explanation for these results is that PKA-C is present in the cilium, but at a concentration lower than that of SMO (and apparently below the limit of detection in their study). Second, in our immunofluorescence microscopy measurements of SMO / PKA-C colocalization in cilia, our ciliary PKA-C immunofluorescence signals are not particularly strong, again suggesting PKA-C is present at only low levels within the cilium. In contrast, endogenous SMO is readily detected in the cilium via immunofluorescence[1,2,11]. Thus, existing data are consistent with the view that levels of SMO can exceed those of PKA-C within the cilium.

When evaluating the relative amounts of SMO and PKA-C in cilia, two additional points are worth noting. First, a significant fraction of the PKA-C in cilia will be bound to PKA-R subunits (K_D < 1 nM)[12,13] in the absence of strong cAMP stimulus, and therefore inactive (i.e., unavailable to phosphorylate GLI.) Indeed, PKA-R subunits are readily detectable within cilia via immunofluorescence and mass spectrometry[6,8]. To block GLI phosphorylation, SMO need only act on the pool of <u>active</u> (non-PKA-R-bound) ciliary PKA-C, not the total population of ciliary PKA-C detected by biochemical or immunofluorescence methods. Therefore, the measurements of total ciliary PKA-C described above will overestimate the size of the PKA-C pool that SMO needs to sequester and inhibit. Second, even maximal levels of Hh pathway activation do not require complete inactivation of PKA-C. According to quantitative mass spectrometry measurements in NIH3T3 cells, stimulation of the Hh pathway with a saturating concentration of SAG21k causes PKA phosphorylation of GLI to drop by only half (from ~25% to ~12.5% of the maximal phosphorylation level, as defined by treatment with the adenylate cyclase agonist forskolin.)[14] A second study obtained a similar result using an independent approach, namely phospho-specific GLI2/3 antibodies to measure levels of GLI phosphorylation in the Hh pathway "off" and "on" states[9]. We conclude that SMO need not be in vast stoichiometric excess of PKA-C to stimulate GLI transcription

via the protein-protein interaction described in our study; even a modest reduction in GLI phosphorylation can suffice to maximally activate GLI transcription.

The question of SMO:PKA-C stoichiometry in cilia is by no means settled, and necessitates further study once better methods for purifying cilia and/or measuring the relevant populations of these proteins become available. Nevertheless, existing work in the field supports our assumption that SMO levels can exceed those of PKA-C specifically in the cilium in the pathway “on” state.

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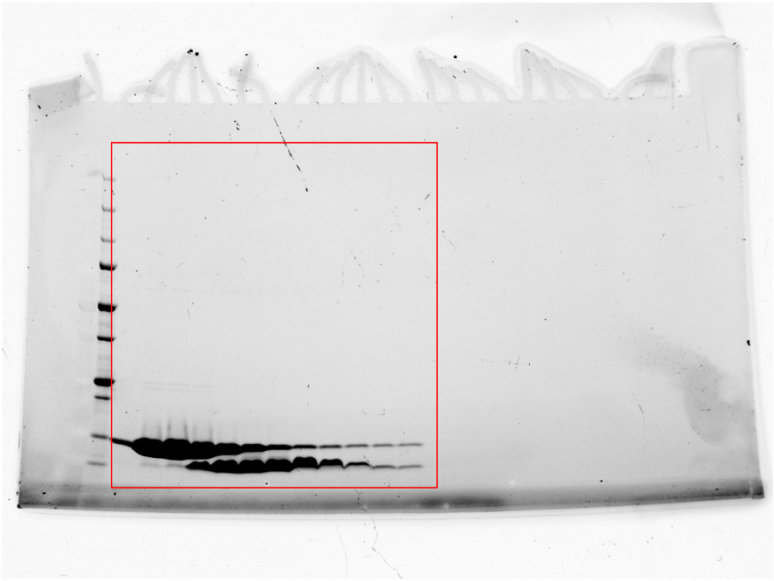
A PKA Inhibitor Motif within Smoothened Controls Hedgehog Signal Transduction

Unprocessed Gels and Blots

Supplementary Figure 1

Total Protein



Source Data for Supplementary Figure 1. Uncropped SDS-PAGE gel.