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Hedgehog induces formation of PKA-Smoothed complexes to promote Smoothed phosphorylation and pathway activation

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Abstract

Hedgehog (Hh) is a secreted glycoprotein that binds its receptor Patched to activate the G protein-coupled receptor-like protein Smoothed (Smo). In *Drosophila*, protein kinase A (PKA) phosphorylates and activates Smo in cells stimulated with Hh. In unstimulated cells, PKA phosphorylates and inhibits the transcription factor Cubitus interruptus (Ci). Here, we found that in cells exposed to Hh, the catalytic subunit of PKA (PKAc) bound to the juxtamembrane region of the C terminus of Smo. PKA-mediated phosphorylation of Smo further enhanced its association with PKAc to form stable kinase-substrate complexes that promoted the PKA-mediated trans-phosphorylation of Smo dimers. We identified multiple basic residues in the C-terminus of Smo that were required for interaction with PKAc, Smo phosphorylation, and Hh pathway activation. Hh induced a switch from the association of PKAc with a cytosolic complex of Ci and the kinesin-like protein Costal2 (Cos2) to a membrane-bound Smo-Cos2 complex. Thus, our study uncovers a previously uncharacterized mechanism for regulation of PKA activity and demonstrates that the signal-regulated formation of kinase-substrate complexes plays a central role in Hh signal transduction.

Introduction

Hedgehog (Hh) signaling controls embryonic development and adult tissue homeostasis. Aberrant Hh signaling contributes to numerous human pathologies, including birth defects and cancer (1-4). The core Hh reception system consists of a twelve-transmembrane protein Patched (Ptc) that binds to Hh and a G-protein coupled receptor (GPCR)-like seven-transmembrane protein Smoothed (Smo), which is required for Hh signal transduction (1, 5, 6). In the absence of Hh, Ptc blocks Smo activity substoichiometrically (7, 8). Binding of Hh to Ptc relieves its inhibition of Smo (9, 10), leading to phosphorylation Smo by multiple kinases (11). Phosphorylation of Smo promotes its accumulation at the cell surface (in

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Drosophila) or primary cilium (in vertebrates) and its reorganization into an active conformation (12-16). Activated Smo activates downstream factors that convert members of the Gli family of zinc finger transcription factors (Cubitus interruptus (Ci) in *Drosophila*) from repressors to activators (11, 17).

Cyclic adenosine 3', 5'-monophosphate (cAMP)-dependent protein kinase A (PKA) plays a phylogenetically conserved role in the inhibition of Hh signal transduction (18-21). In the absence of Hh, PKA phosphorylates multiple sites in Ci and Gli proteins, priming them for phosphorylation by glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1). Phosphorylation targets Ci or Gli for ubiquitination by the β -TRCP family of E3 ubiquitin ligases (Slimb in *Drosophila*) and subsequent proteolysis to generate the repressor forms of Ci or Gli (22-28). PKA-mediated phosphorylation and proteolysis of Ci or Gli is inhibited by Hh stimulation (29-31). In *Drosophila*, PKA can also promote Hh signaling by phosphorylating Smo in response to Hh stimulation (13, 32-34). PKA phosphorylates the C-terminus of Smo at three sites, which primes Smo for phosphorylation by CK1 and GPCR kinase 2 (Gprk2 also known as GRK2) at adjacent sites (13, 33-35). Hyper-phosphorylation of Smo by these kinases promotes its cell surface accumulation and active conformation (13, 15, 32, 35). In vertebrates, PKA does not phosphorylate Smo; instead, CK1 α and Grk2 phosphorylate the C-terminus of Smo at multiple sites, leading to its ciliary accumulation and active conformation (16). The kinesin-like protein Costal2 (Cos2) and its vertebrate homolog Kif7 play a dual role in Hh signal transduction (36-40). In the absence of Hh, Cos2 forms a complex with Ci (Kif7 with Gli in vertebrates) and promotes its proteolytic processing (30, 37-39). In the presence of Hh, Cos2 or Kif7 is required for maximal pathway activation, likely by interacting with the activated Smo (41, 42).

How Hh signaling promotes PKA-dependent phosphorylation of Smo and inhibits PKA-dependent phosphorylation of Ci is poorly understood. PKA exists in two states: as an inactive tetramer of two regulatory (PKAr) and two catalytic (PKAc) subunits and as the dissociated PKAr dimer and two catalytically-active PKAc monomers (43). The equilibrium between these states is controlled by binding of cAMP to PKAr subunits, which promotes the dissociation of the PKA tetramer and thereby, the activity of PKAc (43). One study revealed that Hh stimulates the GPCR activity of Smo that acts through the G protein Gai to decrease cAMP-dependent PKA activity, and thereby inhibit Ci phosphorylation and processing (44). However, a cAMP-independent and constitutively active form of PKAc can substitute for endogenous PKAc to confer normal patterning during wing development, implying that Hh signaling could counteract PKA through mechanisms in addition to the Gai-cAMP pathway (18, 19). Furthermore, Hh-mediated inhibition of Gai- and cAMP-dependent PKA activity does not explain how Hh stimulates phosphorylation of Smo by PKA. Multiple phosphatases, including protein phosphatase 1 (PP1), PP2A, and PP4, are involved in the regulation of Smo phosphorylation (45, 46), but it is unclear whether the activities of these phosphatases are regulated by Hh signaling.

Here, we found that Hh induced the formation of a complex between Smo and PKAc and that the signal-induced PKAc-Smo association promoted phosphorylation of Smo and activation of the Hh pathway. Using a biosensor that monitors PKA activity, we found that Hh signaling increased the activity of PKA at the plasma membrane in a manner that

depended on Smo. Hh promoted the binding of PKAc to the membrane-proximal region of the C-terminal cytoplasmic tail (C-tail) of Smo and phosphorylation of Smo, which led to further recruitment of PKAc to the distal region of the Smo C-tail, forming a positive feedback loop to promote phosphorylation of Smo. Finally, we found that PKAc formed distinct kinase-substrate complexes in the presence or absence of Hh. In the absence of Hh, PKAc bound Cos2 to form a PKAc-Cos2-Ci complex in the cytosol; whereas, stimulation with Hh recruited PKAc to Smo and induced the formation of an active signaling complex containing PKAc, Smo, and Cos2 at the plasma membrane.

Results

Hh signaling regulates the abundance of the PKA catalytic subunit

Previously, we have shown that a cAMP-independent and constitutively active form of murine PKAc, mC*, can substitute for endogenous PKA to confer normal regulation of Hh signaling in wing imaginal discs (18). Here, we expressed mC* uniformly along the anteroposterior (A/P) axis of the dorsal compartment of wing imaginal discs in *Drosophila melanogaster* larvae using a *UAS-mC** transgene driven by a dorsal compartment specific Gal4 driver, *apterous (ap)-gal4 (ap>mC*)*. We noticed that the abundance of mC* (which we visualized using an antibody against PKAc) was increased in cells in the posterior (P) compartment and in cells adjacent to the A/P boundary in the anterior (A) compartment, compared with cells away from the A/P boundary in the A compartment (Fig. 1A; Fig. S1A). mC* abundance in these cells was further increased by overexpression of C-terminally Flag-tagged Smo (Smo-Fg) (Fig. 1B) or by an N-terminally green fluorescent protein (GFP)-tagged Smo (GFP-Smo) (Fig. S1B). Because both the P compartment cells and A compartment cells near the A/P boundary were exposed to Hh ligands produced by the P compartment cells (47, 48), the increased abundance of mC* suggested that mC* could be regulated by Hh. Consistent with this model, coexpression of a *UAS-Hh* transgene with *UAS-mC** and *UAS-Smo-Fg* induced accumulation of mC* and Smo-Fg in A compartment cells away from the A/P boundary (Fig. 1C). To address whether Ptc was involved in the regulation of the abundance of mC*, we created clones of cells homozygous for a null allele of *ptc*, *ptc^{IIW}* (49). We found that the abundance of mC* was increased in *ptc^{IIW}* clones in the A compartment away from the A/P boundary in wing discs with uniformly expressed mC* and Smo-Fg (Fig. 1D). Thus, Hh signaling may increase the abundance of mC* in a manner facilitated by Smo and inhibited by Ptc. Because *ap-gal4* drove the expression of *UAS* transgenes uniformly along the A/P axis (Fig. S1A'), the increased abundance of mC* in the P compartment cells of wing discs expressing *ap>mC** suggests that Hh increased mC* protein stability in this context.

Hh increases PKAc abundance depending on phosphorylation of the Smo C-tail

PKA phosphorylates the C-tail of Smo at three sites (Ser⁶⁶⁷, Ser⁶⁸⁷, and Ser⁷⁴⁰) to transduce the Hh signal (13, 33, 34), suggesting that the C-tail of Smo could be required for the ability of Hh signaling to stabilize mC*. Consistent with this hypothesis, unlike full-length Smo, expression of a truncated Smo lacking its C-tail (Smo- C) did not increase the abundance of mC* (Fig. S1). Moreover, a variant of full-length Smo with the three PKA phosphorylated serines mutated to alanine (SmoSA) also failed to increase the abundance of mC* (Fig. 1E-

E”). In contrast, coexpression of a phosphomimetic and constitutively active form of Smo, SmoSD, which contains serine to aspartate substitutions in the three PKA phosphorylated serines and adjacent CK1 phosphorylated serines (Ser⁶⁷⁰, Ser⁶⁷³, Ser⁶⁹⁰, Ser⁶⁹³, Ser⁷⁴³, and Ser⁷⁴⁶) (13), stabilized mC* in A compartment cells away from the A/P boundary (Fig. 1F), albeit to a lesser degree than in wing discs overexpressing Hh and Smo-Fg (compare Fig. 1F to 1C). In addition, wing discs coexpressing mC* and SmoSD-Fg had increased abundance of mC* in cells of the P compartment cells compared to those in A compartment away from the A/P boundary, despite that the expression of SmoSD-Fg was uniform along the A/P axis (Fig. 1F). Thus, Hh signaling may increase the abundance of mC* through both phosphorylation-dependent and independent mechanisms: Hh-induced phosphorylation of the C-tail of Smo promoted stabilization of mC* but an additional Hh-regulated event was required to confer full stabilization of mC*.

Hh signaling increases the activity of PKA at the plasma membrane

Our results suggested that Hh promotes Smo-dependent stabilization of PKAc. To determine whether Hh signaling increases the activity of endogenous PKAc, we expressed the intramolecular Fluorescence Resonance Energy Transfer (FRET)-based biosensor AKAR3 (A-kinase activity reporter 3) (fig. S2A), which responds to phosphorylation by PKA (50, 51), in cultured *Drosophila* S2 cells. Increasing global PKA activity by exposing cells to forskolin, which activates adenylyl cyclase (52), or by expression of mC* increased AKAR3 FRET (Fig. 2A and fig. 2B). However, AKAR3 FRET was not increased in cells with stimulated with exogenous Hh [For this and subsequent experiments, Hh stimulation refers to cells that were exposed to Hh-conditioned media and transfected with a *UAS-Hh* construct (53).] (Fig. 2A and fig. S2B).

Because Hh increased the abundance of mC* through Smo, we reasoned that it may increase endogenous PKA activity locally at the plasma membrane. Therefore, we fused a myristoylation (Myr) signal to the N-terminus of AKAR3 (Myr-AKAR3) to target it to the plasma membrane (fig. S2A). When transfected into S2 cells, Myr-AKAR3 was mainly localized at the plasma membrane, whereas AKAR3 exhibited diffusive cytoplasmic and nuclear localization (Fig. 2B). Moreover, stimulation with Hh, but not exposure to forskolin or expression of mC*, increased Myr-AKAR3 FRET (Fig. 2C and fig. S2C). Expression of a membrane-tethered mC* (Myr-mC*), but not its kinase dead form (Myr-KD), also increased Myr-AKAR3 FRET (Fig. 2D and fig. S3A).

To determine whether the Hh-induced increase in plasma membrane-associated PKA activity depended on Smo, we knocked down Smo in S2 cells by expressing a double stranded RNA (dsRNA) interference (RNAi) oligonucleotide that targeted the 5' untranslated region (5' UTR) of *smo* (Smo RNAi 5'UTR), cotransfected cells with Myr-AKAR3, and stimulated with Hh. Although Smo knockdown did not alter basal Myr-AKAR3 FRET, it suppressed Hh-induced Myr-AKAR3 FRET (Fig. 2E and fig. S3B), supporting the conclusion that Hh increased membrane-associated PKA activity through Smo.

To examine whether Hh regulated plasma membrane-associated PKA activity under physiological conditions, we generated transgenic flies carrying *UAS-Myr-AKAR3*. We

expressed Myr-AKAR3 in wing imaginal discs using a wing specific Gal4 driver, *MS1096*, which activates *UAS* transgenes uniformly along the A/P axis (54). We found that Myr-AKAR3 exhibited higher FRET in P compartment cells than in A compartment cells (Fig. 2F and fig. S4A). We also generated *ptc^{IIW}* or *smo* [*smo³* (55)] null mutant clones in wing discs expressing *MS1096>Myr-AKAR3* and found that Myr-AKAR3 FRET was markedly increased in A compartment *ptc^{IIW}* mutant clones (Fig. 2G and fig. S4B). In contrast, Myr-AKAR3 FRET decreased in P-compartment *smo³* mutant cells compared with P compartment wild type cells (Fig. 2H and fig. S4C). Thus, Hh signaling through Smo increases PKA activity at the plasma membrane under physiological conditions.

Hh induces the formation of a Smo-PKAc complex that stabilizes PKAc

The observations that Smo stabilized mC* in a Hh-dependent manner and that Hh increased Smo-dependent endogenous plasma membrane-associated PKA activity prompted us to test whether Hh could induce the formation of a complex including Smo and PKAc. Closer examination of wing discs coexpressing mC* and GFP-Smo revealed that PKAc co-localized with GFP-Smo in P compartment cells but not A compartment cells away from the A/P boundary (Fig. 3A-C). We performed coimmunoprecipitation experiments on lysates of S2 cells transfected with a C-terminally YFP-tagged mC* (mC*-YFP) and N-terminally Myc-tagged wild type (Myc-Smo), phosphorylation-deficient (Myc-SmoSA), or phosphomimetic (Myc-SmoSD) form of Smo, followed by stimulation with Hh. Smo is degraded by proteasome in the absence of Hh (56); therefore, we also added the proteasome inhibitor MG132 to cells four hours before lysis. To minimize the possibility of potential unregulated protein interactions due to overexpression, we expressed mC*-YFP at a low abundance by transfecting cells with small amounts of the *UAS-mC*-YFP* DNA construct. Myc-Smo coimmunoprecipitated mC*-YFP in lysates of Hh-stimulated, but not unstimulated, cells (Fig. 3D). Moreover, Myc-SmoSD formed a complex with mC*-YFP and Myc-SmoSA failed to bind mC*-YFP in the presence or absence of Hh stimulation (Fig. 3D). Because PKA also interacts with Ci in a complex with Cos2 (30), we repeated coimmunoprecipitation experiments in Cl8 cells, which express endogenous Ci ((57) and found that, similar to that in S2 cells, the interaction between Smo and PKA in Cl8 cells depended on Hh stimulation and the PKA phosphorylated residues in Smo (fig. S5A).

To determine whether Smo could form a complex with endogenous PKAc in response to Hh stimulation, we exposed S2 or Cl8 cells to forskolin to increase the abundance of free PKAc. In the absence of forskolin, Myc-Smo did not coimmunoprecipitate endogenous PKAc, even in cells stimulated with Hh (Fig. S5B). However, in cells exposed to forskolin, PKAc coimmunoprecipitated with Myc-Smo in the presence, but not in the absence of Hh stimulation (Fig. 3E and fig. S5B-C). Myc-SmoSA did not coimmunoprecipitate PKAc in the presence of Hh stimulation; whereas, Myc-SmoSD formed a complex with endogenous PKAc, even in the absence of Hh stimulation (Fig. 3E; fig. S5C).

To determine whether Hh-induced association of Smo and PKAc stabilizes PKAc, we measured the half-life of mC*-YFP in S2 cells cotransfected with mC*-YFP and Myc-Smo, Myc-SmoSA, or Myc-SmoSD. We also cotransfected Myc-CFP as an internal control. We grew cells with or without Hh-stimulation for 24 hours, then exposed them to cycloheximide

(CHX) for different lengths of time to block protein synthesis and measured the abundance of mC*-YFP by Western blot analysis. In cells expressing Myc-Smo and mC*-YFP, Hh stimulation extended the half-life of mC*-YFP from about four to greater than eight hours (Fig. 3F). However, in cells expressing Myc-SmoSA and mC*-YFP, Hh stimulation had no effect on the turnover of mC*-YFP (Fig. 3G). Consistent with our observations in vivo (Fig. 1F), coexpression of Myc-SmoSD increased the half-life of mC*-YFP compared to coexpression with Myc-Smo, and Hh stimulation further increased this effect (Fig. 3, F and H). Thus, stimulation with Hh may stabilize PKAc through its association with Smo, and the interaction between Smo and PKAc is likely regulated by the phosphorylation of Smo C-tail.

PKAc interacts with multiple regions in the Smo C-tail

To begin to identify the PKAc-binding site(s) in Smo, we examined the colocalization of Smo and mC*. We cotransfected S2 cells with C-terminally CFP-tagged full-length wild-type Smo (SmoWT) or Smo with different deletions of regions of the C-tail (Fig. 4A) and mC*-YFP and monitored protein localization by confocal microscopy. Full-length Smo-CFP colocalized with mC*-YFP when both were highly overexpressed (Fig. 4A), presumably because mC* phosphorylated the C-tail of Smo. Deletion of the C-terminus of Smo up to amino acid (aa) 650 (Smo C818, Smo C730, Smo C661, and Smo C650) did not perturb colocalization of Smo-CFP and mC*-YFP (Fig. 4A). Smo-CFP with larger deletions of the Smo C-terminus (Smo C630 and Smo C570) localized to the plasma-membrane, but did not colocalize with mC*, which appeared diffuse throughout the cell (Fig. 4A), suggesting that the region from aa 630 to 650 may harbor a PKAc binding site(s). However, deleting this region in the context of full-length Smo (Smo 625-678) did not affect its colocalization with mC* (Fig. 4A), suggesting that in the absence of aa 630 to 650, other region(s) may also mediate the colocalization of Smo and mC*. The C-terminal region from aa 818 to 1035 mediates binding of Cos2 to Smo in the presence of Hh (41); however, deleting this region in Smo 625-678 (Smo C818) did not affect its colocalization with mC* (Fig. 4A). Moreover, deleting the Smo auto-inhibitory domain [SAID, (15), Smo 661-818, (Fig. 4B)], did not affect its colocalization with mC* (Fig. 4A). However, a larger deletion from aa 630 to 818 (Smo 630-818) abolished colocalization Smo and mC* (Fig. 4A), suggesting that multiple domains within this region may mediate the interaction between Smo and PKAc.

To identify whether regions of Smo that were important for its colocalization with mC* were required for direct binding of Smo to PKAc, we performed in vitro binding assays with glutathione-S-transferase (GST)-tagged Smo or Smo variants and recombinant PKAc. We found that multiple GST-fusion proteins corresponding to regions of the Smo C-tail (aa556-650, aa651-818, aa601-700, aa700-748, or aa748-818) coprecipitated recombinant PKAc (Fig. 4C). In contrast, GST alone or GST-fusion proteins containing regions of Smo located C-terminal to the SAID domain (aa808-899, aa899-937, or aa818-1035) did not coprecipitate PKAc (Fig. 4C). These results support the conclusion that multiple PKAc-binding sites between Smo aa 630 and 818 could mediate its interaction with PKAc.

We noticed that the juxtamembrane region of the C-tail of Smo contains multiple clusters of basic amino acids, which we named R1', R2', and R3' (Fig. 4B). Because consensus sites (R/K R/K × S/T) for PKAc-mediated phosphorylation contain clusters of basic amino acids

that are involved in kinase-substrate interaction (58), we hypothesized that these clusters in Smo might be critical for recruiting PKAc. Therefore, we mutated basic amino acids to alanine (RA) in one, two, or all three clusters and performed in vitro binding assays with GST-tagged Smo or mutant Smo and recombinant PKAc. Mutating cluster R2' or R3' (RA2' or RA3') reduced, whereas mutating clusters R2' and R3' (RA2'3') or mutating all three clusters (RA1'2'3') nearly abolished the ability of GST-Smo556-650 to coprecipitate PKAc (Fig. 4, B and C). We also identified clusters of basic amino acids in the SAID domain (fig. S6A) and found that mutating these residues to alanine reduced the ability of GST-Smo700-748 to coprecipitate PKAc (fig. S6B). Thus, multiple clusters of basic amino acids may facilitate the interaction between Smo and PKAc.

Hh promotes the binding of PKAc to the juxtamembrane region of the C-tail of Smo

To address whether Hh stimulation could promote the association of PKAc with the juxtamembrane region of the C-tail of Smo, we analyzed the colocalization of mC*-YFP and Smo-CFP with deletions of the Smo C-tail in the presence or absence of Hh. Although Smo C630 did not colocalize with mC* in the absence of Hh, Hh stimulation resulted in colocalization of these proteins (Fig. 4D). In contrast, Smo C570 did not colocalize with mC* in the presence or absence of Hh stimulation (Fig. 4D). Smo C650-CFP with mutations in the R3' cluster (Smo C650RA3') colocalized with mC* in the presence, but not the absence, of Hh stimulation (Fig. 4D). In contrast, mutating all three clusters in Smo C650-CFP (Smo C650RA1'2'3') abolished Hh-induced colocalization with mC*. Truncated Smo variants could indirectly recruit mC* through dimerization with endogenous Smo in cells stimulated with Hh (15); however, the Hh-induced colocalization of mC*-YFP with Smo C630-CFP or Smo C650RA3'-CFP was not affected in cells with knockdown of endogenous Smo by Smo RNAi 5'UTR (fig. S7).

To determine whether Hh induced direct binding of PKAc to the juxtamembrane region of the C-tail of Smo, we performed FRET analysis (15, 59) on S2 cells transfected with mC*-YFP and deletion mutants of Smo-CFP. Stimulation with Hh increased mC*-YFP FRET with Smo C630-CFP, but not Smo C570-CFP, compared to unstimulated cells (Fig. 4E and fig. S8). mC*-YFP and Smo C650-CFP showed high basal FRET that was reduced by mutation of the R3' cluster (Fig. 4E and fig. S8). Stimulating cells with Hh induced mC*-YFP FRET with Smo C650RA3'-CFP; however, Smo C650RA1'2'3'-CFP did not show basal or Hh-induced FRET with mC*-YFP (Fig. 4E and fig. S8). Thus, stimulation with Hh may expose a binding pocket in the juxtamembrane region of the C-tail of Smo that uses multiple clusters of basic amino acids to recruit PKAc.

Inhibition of Hh-induced binding of PKAc to Smo blocks trans-phosphorylation of Smo and activation of Hh signaling

The identification of residues in Smo that mediated Hh-induced recruitment of PKAc provided an opportunity to assess the biological importance of the interaction between PKAc and Smo in the regulation of Smo phosphorylation and Hh signaling. Compared to wild-type full-length Myc-Smo, Myc-SmoRA2'3' or Myc-SmoRA1'2'3' showed a drastically reduced Hh-dependent interaction with endogenous PKAc in S2 cells exposed to forskolin (Fig. 5A). However, stimulation with Hh increased the phosphorylation of Myc-

Smo detected with a phosphorylation-specific antibody that recognizes phosphorylated PKA site 2 (Ser⁶⁸⁷) and adjacent CK1 sites (Ser⁶⁹⁰ and Ser⁶⁹²) in the C-tail of Smo (32), and RA2'3' or RA1'2'3' mutations in Smo reduced but did not abolish Hh-induced phosphorylation of Smo (Fig. 5B). Consistent with this observation, we found that compared to Myc-Smo, expression of Myc-SmoRA2'3' or Myc-SmoRA1'2'3' reduced but did not abolish the ability of Hh to stimulate activation of a *ptc-luc* reporter in C18 cells (Fig. 5C). Thus, clusters of basic amino acids in the C-tail of Smo were required for binding PKAc but mutation of these residues did not completely block PKAc-mediated phosphorylation of Smo or activation of Hh signaling.

Our previous study indicates that Smo forms a constitutive dimer (15), and a recent study shows that Hh stimulation can promote higher order oligomers of Smo (60). Therefore, we speculated that Hh-induced phosphorylation of Smo and activation of the Hh signaling reporter in cells expressing Myc-SmoRA2'3' and Myc-SmoRA1'2'3' could be due to the interaction between exogenous mutant Smo and endogenous Smo. We assessed the ability of Hh to induce Smo phosphorylation and reporter activity in cells with knockdown of endogenous Smo and expressing Myc-Smo or its mutant derivatives. To avoid knocking down exogenous Smo, we expressed Smo RNAi 5'UTR, which targeted the 5' UTR of *smo* that was absent in Smo-encoding transgenes. The inhibition of Hh-induced phosphorylation of Myc-Smo by mutation of clusters R2' and R3' or R1', R2', and R3' was greater in S2 cells with knockdown of endogenous Smo than in cells without (Fig. 5, D and E). Moreover, the induction of the *ptc-luc* reporter in Hh-stimulated C18 cells expressing Myc-SmoRA2'3' or Myc-SmoRA1'2'3' was greatly reduced in cells with knockdown of endogenous Smo (Fig. 5, C and E).

We hypothesized that the inability of SmoRA2'3' and SmoRA1'2'3' to activate Hh signaling could be because they were not effectively phosphorylated by PKA. To assess whether phosphomimetic mutations in the C-tail of Smo could rescue signaling defects of Smo with mutations in the clusters of basic amino acids, we mutated serine to aspartate at the three clusters of PKA and CK1 phosphorylation sites (13) in Myc-SmoRA2'3' and Myc-SmoRA1'2'3' to generate Myc-SmoSDRA2'3' and Myc-SmoSDRA1'2'3'. We expressed phosphomimetic mutant Smo proteins in C18 cells with knockdown of endogenous Smo and found that compared to SmoRA2'3' or SmoRA1'2'3', SmoSDRA2'3' and SmoSDRA1'2'3' caused a marked increase in the basal and Hh-induced activation of the Hh signaling reporter (Fig. 5E). Previous studies show that Smo transduces the Hh signal by interacting with Cos2 (41, 53, 61-63). Consistent with its reduced ability to promote Hh-induced reporter activation, Myc-SmoRA1'2'3' coprecipitated less Cos2 than Myc-Smo (Fig. 5F). In contrast, phosphomimetic Myc-SmoSDRA1'2'3' and Myc-SmoSD efficiently coprecipitated Cos2 (Fig. 5F).

The observation that Hh stimulation induced the phosphorylation of Myc-SmoRA1'2'3' in the presence of endogenous Smo could indicate that PKAc that was bound to endogenous Smo phosphorylated Myc-SmoRA1'2'3' in trans, or that endogenous Smo that was directly phosphorylated by PKAc in cis coprecipitated with Myc-SmoRA1'2'3'. To distinguish between these possibilities, we coexpressed Myc-SmoRA1'2'3', which did not bind PKAc (Fig. 5A) but had wild-type PKA phosphorylation sites and thus could be phosphorylated

(Fig. 5B), and Flag-tagged SmoSD (Fg-SmoSD), which bound PKAc (Fig. 3E) but had mutant PKA phosphorylation sites in S2 cells with knockdown of endogenous Smo. As a negative control, we coexpressed Myc-SmoRA1'2'3' with Flag-tagged SmoSA (Fg-SmoSA), which did not bind PKAc (Fig. 3D-E) in S2 cells with knockdown of endogenous Smo. In transfected cells stimulated with Hh, coexpression of Fg-SmoSD, but not Fg-SmoSA, resulted in the phosphorylation of Myc-SmoRA1'2'3' (Fig. 5G), suggesting that Hh-induced Smo-PKAc complexes can engage in trans-phosphorylation of Smo oligomers. Of note, although SmoSD exhibited constitutive binding to PKAc in cells expressing mC* or exposed to forskolin (Fig. 3, D and E), in physiological conditions the binding of PKAc to SmoSD could depend on Hh because endogenous free PKAc may be present at limiting concentrations, which could explain why coexpression of Fg-SmoSD did not result in phosphorylation of Myc-SmoRA1'2'3' in the absence of Hh stimulation (Fig. 5G).

PKAc-binding deficient Smo variants exhibit signaling defects in vivo

To explore the biological relevance of the Hh-induced interaction between Smo and PKAc, we examined the abundance of Ci and proteins encoded by the Hh target genes *ptc* and *en* (*engrailed*) in wing imaginal discs expressing PKAc-binding deficient Smo variants. Stabilization of Ci requires lower amounts of Hh signaling activity, whereas activation of the transcription of *ptc* requires intermediate and *en* requires high thresholds of Hh signaling activity (64). We generated *smo*³ clones in the absence or presence of transgenic expression of Myc-tagged wild-type Smo (Myc-Smo), Myc-SmoRA2'3', or Myc-SmoRA1'2'3' using one of two Gal4 drivers *tub-Gal4* or *C765*. Immunofluorescence staining with an antibody targeting Smo indicated that *C765* drives (*C765>Myc-Smo*) expression slightly higher and *tub-Gal4* drives *UAS-Myc-Smo* (*tub>Myc-Smo*) expression much higher than the endogenous *smo* locus (Fig. S9A-C). Consistent with previous findings (55), *smo*³ clones located near the A/P boundary had no detectable immunoreactivity for Ptc and En (Fig. 6, A and B). In addition, *smo*³ clones near the A/P boundary had reduced immunoreactivity for full-length Ci (Fig. 6A), consistent with the observations that Smo is required for stabilization of full-length Ci in response to stimulation by endogenous Hh (65). As expected, *tub>Myc-Smo* completely rescued the immunoreactivity for Ptc, En, and Ci in *smo*³ clones located near the A/P boundary (Fig. 6, C and D). In contrast, *tub>Myc-SmoRA2'3'* did not rescue immunoreactivity for En (Fig. 6 E), but partially rescued immunoreactivity for Ptc (Fig. 6, E and F) in *smo*³ clones at the A/P boundary. Ci was fully stabilized in *smo*³ clones with *tub>Myc-SmoRA2'3'* at the A/P boundary (Fig. 6E), suggesting that SmoRA2'3' cannot convert Ci into the active but labile form in response to Hh (66). We found similar results with *tub>Myc-SmoRA1'2'3'* (Fig. 6, G and I), suggesting that both Smo variants can transduce low amounts of Hh signaling when highly expressed. In contrast, unlike wild-type Smo, which fully rescued immunoreactivity for Ptc in *smo*³ clones (fig. S9D), neither Myc-SmoRA2'3' nor Myc-SmoRA1'2'3' rescued immunoreactivity for Ptc in *smo*³ clones at the A/P boundary when expressed at lower amounts using the *C765-Gal4* driver (Fig. 6, I and J). However, both *C765>Myc-SmoRA2'3'* and *C765>Myc-SmoRA1'2'3'* slightly increased immunoreactivity for Ci in *smo*³ clones at the A/P boundary (Fig. 6, I and J), suggesting that these Smo variants have at least a minimal ability to stimulate pathway activity. Thus, PKAc binding to Smo likely plays an important role in Hh pathway activation in vivo.

PKAc-binding deficient Smo fails to stabilize PKAc

Our data suggested that Hh stabilizes PKAc through Smo and showed that SmoSD bound and stabilized PKAc in the absence of Hh stimulation (Fig. 3F-H). To distinguish whether the effect of Smo on PKAc stabilization was due to the interaction of Smo and PKAc or to an indirect mechanism that occurred downstream of Smo activation, we sought to uncouple PKAc stabilization from Smo activation. Although both Myc-SmoSD and Myc-SmoSDRA1'2'3' activated the Hh reporter in C18 cells with knockdown of endogenous Smo (Fig. 5E), Myc-SmoSDRA1'2'3' coprecipitated less mC*-YFP than Myc-SmoSD (Fig. S10A). Although Myc-SmoSDRA1'2'3' exhibited some ability to promote the stability of mC*-YFP compared with wild type Smo, Myc-SmoSDRA1'2'3' was less effective at stabilizing mC*-YFP than Myc-SmoSD (Fig. S10B-C).

We found that clusters of basic amino acids in the SAID domain of Smo were important for its binding to PKAc (Fig. S6B). Therefore, we tested whether deletion of the SAID domain in Myc-SmoSDRA1'2'3' would further reduce its ability to stabilize mC*. Myc-Smo SAIDRA1'2'3' did not coprecipitate (fig. S10A) or stabilize (Fig. S10E) mC*-YFP in S2 cells. However, Smo SAIDRA1'2'3' activated the Hh reporter in the absence of endogenous Smo or Hh stimulation in C18 cells (Fig. S10D), suggesting that the interaction between Smo and PKAc rather than activation of Smo was required for the stabilization of PKAc.

Hh induces a switch in PKAc association with Cos2-Ci to Smo-Cos2 complexes

Our previous studies show that PKAc and Ci form a complex scaffolded by Cos2 and that formation of this complex is inhibited by Hh signaling (30, 41). To explore the dynamic change of PKAc-substrate complexes in Hh signaling “off” and “on” states, we monitored the subcellular localization of PKAc, Ci, Cos2, and Smo in S2 cells. Because Ci is proteolytically processed after phosphorylation by a complex of PKAc and Cos2 (30), we used a phosphorylation and proteolysis deficient form of Ci, Ci^{-PKA}, which contained serine to alanine mutations in the three PKA phosphorylation sites required for processing of Ci (54) and thus enabled us to observe stable complexes of PKAc, Cos2, and Ci. We cotransfected cells with mC*-YFP, Myc-Smo, CFP-tagged Ci^{-PKA} (Ci^{-PKA}-CFP), and Flag-tagged Cos2 (Fg-Cos2). In the absence Hh, Ci^{-PKA}-CFP and mC*-YFP colocalized with Fg-Cos2, but not Myc-Smo, in intracellular puncta (Fig. 7A). In contrast, in the presence of Hh stimulation, mC*-YFP and Fg-Cos2 colocalized with Myc-Smo at the plasma membrane; whereas, Ci^{-PKA}-CFP had a diffuse localization pattern (Fig. 7A). Although Ci^{-PKA}-CFP and mC*-YFP colocalized in the absence of Hh (Fig. 7A), we did not detect FRET between them (Fig. 7B and fig. S11), suggesting that their association is indirect and consistent with the observation that Cos2 functions as a scaffold to bring Ci and kinases together (30).

We hypothesized that Hh-induced colocalization of PKAc and Cos2 at the plasma membrane was mediated by Smo rather than a direct protein interaction. We performed FRET analysis on S2 cells transfected with mC*-YFP and CFP-tagged Cos2 (Cos2-CFP) in the absence or presence of Myc-Smo. In the absence of Hh and regardless of the presence or absence of Myc-Smo, Cos2-CFP and mC*-YFP colocalized in intracellular puncta (fig. S11) and exhibited high FRET (Fig. 7B). In cells stimulated with Hh, the FRET between Cos2-

CFP and mC*-YFP was decreased (Fig. 7B), although Cos2-CFP and mC*-YFP colocalized at the plasma membrane (Fig. S11), suggesting that the interaction between Smo and PKAc may mediate the interaction between Cos2 and PKAc.

To determine if PKAc directly interacts with Smo in the Hh-induced PKAc-Smo-Cos2 complex, we examined the FRET between mC*-YFP and Smo-CFP^{L3}, which contains CFP inserted into the third intracellular loop of full-length wild-type Smo (15). In S2 cells expressing Fg-Cos2, mC*-YFP had little colocalization with Smo-CFP^{L3} (Fig. S11) and did not show significant FRET with Smo-CFP^{L3} (Fig. 7B). In contrast, stimulating cells with Hh induced high FRET (Fig. 7B) and colocalization between Smo-CFP^{L3} and mC*-YFP at the plasma membrane and in intracellular punta (Fig. S11). In combination with the observation that Hh reduced the FRET between Cos2-CFP and mC*-YFP, these results suggest that PKAc directly interacts with Smo instead of Cos2 in the Hh-induced PKAc-Smo-Cos2 complex.

Discussion

Phosphorylation plays a central role in Hh signal transduction both in *Drosophila* and vertebrates (11, 17). Understanding how Hh regulates the phosphorylation of individual pathway components is complicated by the fact that the same kinases, such as PKA and CK1, can phosphorylate both upstream and downstream signaling components. Moreover, these phosphorylation events are regulated in opposite directions in the “on” and “off” states of Hh signaling, making it unlikely that global regulation of PKA and CK1 kinase activities could be the major mechanism for pathway regulation. Here, we provided evidence that Hh induces a switch from an intracellular PKAc-Cos2-Ci complex that favors phosphorylation of Ci to a plasma membrane associated PKAc-Smo-Cos2 complex that favors phosphorylation of Smo. Thus, our study suggests that Hh switches PKA substrate selection by controlling the formation of distinct PKAc-substrate complexes (Fig. 7C).

The identification of PKA as an inhibitory component of the Hh signaling pathway raised the possibility that the Hh signal is transduced by the GPCR activity of Smo to inhibit cAMP-dependent PKA kinase activity (18-20). In both mammalian and *Drosophila* cultured cells, overexpressed Smo activates G α i to inhibit cAMP, and inactivation of G α i alters the expression of Hh target genes under certain conditions (44, 67, 68). Using a biosensor (AKAR3) that measures PKA activity, we found that Hh stimulation did not alter the overall intracellular PKA activity (Fig. 2), consistent with an earlier finding that inhibition of Hh signaling by overexpression of Ptc in wing imaginal discs does not alter the activity of PKA measured by kinase assay (19). Instead, we found that Hh signaling increased the activity of PKA at the plasma membrane in both cultured cells and wing imaginal discs (Fig. 2). By colocalization, FRET, and coimmunoprecipitation assays, we showed that Hh induces the interaction of Smo and PKAc at the plasma membrane. Furthermore, we showed that Hh signaling recruits PKAc to the juxtamembrane region of the C-tail of Smo and that Hh-induced PKAc binding to this region is critical for the phosphorylation of Smo and activation of the Hh pathway (Figures 4-6). Although it remains possible that Hh could act through the GPCR activity of Smo to regulate a local pool of PKA activity to influence Ci

phosphorylation (44), our study suggests that Hh also induces the formation of a PKAc-Smo complex at the plasma membrane to transduce Hh signals.

Recent studies in mammalian systems reveal that GPCRs and Gas may regulate activity of the Sonic hedgehog (Shh) pathway through cAMP and PKA (69, 70). For example, inactivation of the GPCR Gpr161 increases Hh signaling in the neural tube, whereas the steady state activity of Gpr161 represses Hh signaling by increasing the concentration of cAMP (69). Furthermore, Gpr161 localizes to primary cilia in the absence of Hh but moves out of cilia in cells exposed to Hh or synthetic Smo agonists (69). Thus, Gpr161 may maintain low basal activity of the Shh pathway by generating local cAMP and PKA activity to promote Gli processing, and Shh and Smo may influence local cAMP and PKA activity indirectly by regulating the ciliary localization of Gpr161 (69). In *Drosophila*, loss of the Gprk2 augments cAMP abundance and Ci processing (71), implying that other GPCRs in addition to Gpr161 may control the steady-state abundance of cAMP and activity of PKA. Although we cannot rule out the possibility that Hh could influence local cAMP concentration and activity of PKA indirectly through regulating another GPCR, our study suggests that Hh-induced increase in the plasma membrane associated activity of PKA is at least in part due to its association with Smo. Because PKAc-mediated phosphorylation further stabilizes Smo, positive feedback regulation may lead to the formation of stable PKAc-Smo complexes.

The subcellular localization of PKA is regulated by A-kinase anchoring proteins (AKAPs), which function as molecular scaffolds that bind and anchor PKA holoenzymes and other enzymes to different subcellular compartments where these enzymes are poised to respond to second messengers (72). In mammalian cells, enrichment of PKA at the basal body of the primary cilium is likely mediated by AKAPs (21, 73). Coupled with the influence of the ciliary localization of Gpr161 on the activity of PKA, AKAP-mediated subcellular compartmentalization of PKA likely generates abundant PKA activity at the base of the cilium, where PKA is essential for Gli phosphorylation and processing (21, 69). *Drosophila* Hh signaling does not require the primary cilium (74); however, it is possible that PKA holoenzymes are targeted by AKAPs to the subcellular compartments containing Smo and Ci. We observed that Hh promoted the formation of Smo-PKAc complexes and the dissociation of Ci-Cos2-PKAc complexes. The dynamic formation of these PKAc-substrate complexes is unlikely to be mediated by AKAPs because AKAPs anchor the PKA holoenzyme through binding to the PKAr subunits (72). In contrast, we showed that Smo and Cos2 interacted with constitutively active PKAc (mC*) that does not bind PKAr, and FRET analysis suggested that these interactions were direct. Moreover, we found that forskolin, which increases the intracellular concentration of cAMP and dissociates PKAc from PKAr, enhanced the binding of Smo to PKAc, and that purified GST-Smo fusion proteins coprecipitated recombinant PKAc. Hence, our study identified Hh signal-regulated PKAc-substrate interactions as an additional layer of PKA regulation. Given the large number of proteins phosphorylated by PKA in a wide variety of cellular processes, we speculate that signal-regulated PKAc-substrate interactions could be a general mechanism used by other molecular pathways.

The interaction between PKAc and Smo was mediated by multiple clusters of basic amino acids in the juxtamembrane region of the C-tail and in the SAID domain of Smo. PKAc recognizes its substrates through ionic interactions between several acidic residues in its kinase domain and the basic residues located two and three residues N-terminal to the phosphorylated residue in its consensus site (R/K R/K $\times$ S/T) (75). Similarly, PKAc binds an endogenous protein kinase inhibitor (PKI) through a cluster of basic residues and an adjacent motif that increases its binding affinity (75). Unlike the high affinity binding between PKAc and PKI, which “locks” the PKA catalytic site, the interaction between PKAc and its substrates is generally transient. Therefore, we speculate that the interaction between PKAc and individual clusters of basic amino acids in the C-tail of Smo is likely a low affinity interaction. However, the presence of multiple clusters of basic residues in each Smo C-tail and the ability of Smo C-tails to oligomerize may enable cooperative binding that would greatly increase the probability of association between PKAc and Smo and thus prevent PKAc from diffusing away once it docks onto the C-tail of Smo. Because of the low abundance of free PKAc in normal cells (19), the stoichiometry of PKAc in the Smo signaling complex is likely to be low. However, several mechanisms may enable efficient phosphorylation of Smo by PKAc: First, PKAc could engage in trans-phosphorylation after binding to Smo. Second, Hh induces the formation of high-order Smo multimerization (60), which would enable PKAc-mediated phosphorylation to quickly spread among individual monomers within Smo multimers. Third, phosphorylation of the C-tail of Smo could further facilitate PKAc recruitment, forming a positive feedback loop to promote processive phosphorylation of Smo.

How does Hh regulate the interaction of Smo and PKAc? Using colocalization and FRET analysis, we observed that Hh induced the binding of mC* to C-terminally truncated forms of Smo including the juxtamembrane PKAc-binding regions (Fig. 4). Access to this region could be hindered due to its closed proximity to the intracellular loops of Smo or the plasma membrane. Hh could induce a conformational change in the Smo transmembrane helices, similar to those caused by ligand binding to GPCRs, to expose the juxtamembrane PKAc-binding region. An alternative, but not mutually exclusive possibility, is that PKAc binding to Smo could be stimulated by Hh-induced Smo oligomerization that would confer cooperativity among individual PKAc binding regions. Hh-induced PKAc binding to Smo was required for its phosphorylation (Fig. 5D) and thus, the physical interaction between PKAc and Smo likely precedes extensive phosphorylation of Smo. However, mutating the three PKA phosphorylation sites (SmoSA) in the C-tail of Smo completely blocked Hh-induced binding to PKAc (Fig. 3), suggesting that basal phosphorylation of Smo may be important to initiate its interaction with PKAc. Our previous study indicated that phosphorylation of Smo by PKA and CK1 induces a conformational switch of the C-tail of Smo from a “closed” conformation, in which the C-terminus of the Smo-C-tail is in close contact with the third intracellular loop (L3), to an “open” conformation, in which the Smo C-terminus moves away from the L3, (Fig. 7C)(15). Thus, mutation of the PKAc phosphorylation sites to non-phosphorylatable alanine could “lock” Smo in a closed conformation that blocks PKAc access; whereas, basal phosphorylation or mutation of the PKAc phosphorylation sites to phosphomimetic aspartate could cause Smo to transiently adopt an open conformation that enables PKAc access. The phosphorylation-induced switch

in the conformation of Smo could also expose the PKAc-binding sites in the SAID domain to facilitate PKAc recruitment. Interestingly, a similar mechanism has been proposed to account for Shh-stimulated phosphorylation of mammalian Smo. Shh induces binding of CK1 α to the membrane proximal region of the C-tail of mammalian Smo to initiate its phosphorylation and phosphorylation of Smo subsequently recruits CK1 α and GRK2 to amplify or maintain phosphorylation of Smo (16). Hence, even though different sets of kinases phosphorylate Smo in *Drosophila* and vertebrates, the mechanisms underlying the regulation of Smo phosphorylation and activation are similar. Because Smo is an attractive target for cancer drug development, understanding how Smo phosphorylation and activity are regulated could open up new avenues for cancer prevention and therapeutics.

Materials and Methods

Drosophila husbandry and genetics

All flies were raised on standard yeast and molasses-based food at 25 °C. *UAS-mC**, *UAS-Smo-Fg*, *UAS-SmoSA-Fg*, *UAS-SmoSD-Fg*, *UAS-GFP-Smo*, *USA-GFP-Smo*, *Ci^{-PKA}*, and *UAS-Hh* have been described (13, 54, 62). *MS1096*-, *C765*-, *tub*-, and *ap-Gal4* drivers have been described (18, 35, 54, 76, 77). C-terminally CFP-tagged wild type or C-terminally truncated Smo variants and Smo-CFP^{L3} have been described (15). *mC*-YFP* and *Ci^{-PKA}-CFP* contain C-terminally fused YFP and CFP, respectively. *Myr-mC** and *Myr-AKAR3* contain an N-terminally fused Myr signal from *Drosophila* Src (78, 79). *Myr-KD* is derived from *Myr-mC** with a point mutation (K72R) in the ATPase domain that inactivates the kinase activity (80, 81). Amino acid substitutions were generated by PCR-based site directed mutagenesis. To generate GST-Smo fusion constructs, DNA fragments encoding different Smo C-terminal regions with either wild-type sequences or amino acid substitutions were amplified by PCR and inserted between *Sall* and *NotI* sites of the *pGEX-4T-1* vector. For generating flies with transgenes inserted at the 75B1 *attP* locus (82), the coding regions for Myc-tagged Smo variants were subcloned between *NotI* and *XhoI* sites of a modified *pUAST* vector with an *attB* sequence inserted upstream of the *UAS*-binding sites (83). All plasmids were verified by complete sequencing of the open reading frame for the gene of interest. Transgenic flies were generated by P-element mediated transformation (84).

Generation of mutant clones

Mutant clones were generated by standard FLP(flippase) /FRT(flippase recognition target)-mediated mitotic recombination (Fig. 1D and Fig. 2, G and H) or the MARCM (Mosaic analysis with a repressible cell marker) system (Fig. 6 and fig.S9) as previously described (18, 77) using the following genotypes: *ptc* mutant clones expressing *mC** and *Smo-Fg* (*yw hs-flp MS1096; FRT42D ptc^{IIW}/FRT42D hs-Myc-GFP; UAS-mC* UAS-Smo-Fg*). *ptc* mutant clones expressing *Myr-AKAR3* (*y hs-flp MS1096; FRT42D ptc^{IIW}/FRT42D hs-CD2; UAS-Myr-AKAR3*). *smo* clones expressing Smo transgenes using *tub-Gal4* (*yw hs-FLP UAS-GFP; tub-Gal80 FRT40/smo³ FRT40; tub-Gal4/UAS-Myc-Smo, Myc-Smo-RA2'3', or Myc-Smo-RA1'2'3'*). *smo* mutant clones expressing *Myr-AKAR3* (*y hs-flp MS1096; smo³ FRT40/ hs-CD2 FRT40; UAS-Myr-AKAR3*). *smo* clones expressing Smo transgenes using

C765 (yw hs-FLP; tub-Gal80 FRT40/smo³ FRT40; C765/UAS-Myc-Smo, Myc-Smo-RA2'3', or Myc-Smo-RA1'2'3').

Cell culture, transfection, immunostaining, immunoprecipitation, in vitro binding assays, and Western blot analysis

Drosophila S2 cells were cultured in Schneider's *Drosophila* Medium (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100 mg/ml of streptomycin at 24°C. C18 cells were cultured in Shields and Sang M3 insect medium (Sigma, S3652) with 2.5% fetal bovine serum, 2.5% fly extract, 0.125 IU/ml (0.5mg/ml) insulin (Sigma, I6634), 100 U/ml of penicillin, and 100mg/ml of streptomycin at 24°C. Transfection was carried out using the Calcium Phosphate Transfection Kit (K2780-01, Invitrogen). For transient gene expression, the corresponding *UAS* constructs were cotransfected with a *ubiquitin-Gal4 (ub-Gal4)* construct as previously described (29). Hh-conditioned medium was collected from HhN-S2 cells induced with 0.7 mM CuSO₄ in the absence of hygromycin B for 24 hours (57). Transfected S2 cells were exposed with Hh-stimulated for 24 hours. Hh-conditioned medium was used at a 6:4 dilution ratio by fresh medium. For maximal Hh signaling efficiency, a *UAS-Hh* construct was also included in the transfection in all experiments where Hh-conditioned media were applied. Forskolin (7.5 μM) was applied for 1 hour before cell collection.

For immunostaining analysis, S2 cells were fixed in 4% formaldehyde and permeabilized in 0.2% Triton X-100. The cells were incubated in primary and secondary antibody for 1.5 hours. Images were captured by confocal microscopy and signals were quantified by ImageJ software. For Smo-PKAc colocalization experiments in Fig. 4, S2 cells were transfected with 5 μg of mC* construct and 5 μg of Smo constructs.

For immunoprecipitation analysis, cell lysates were incubated with primary antibody overnight at 4 °C. To collect immunoprecipitated protein complexes, 60 μl of a 50% protein-A agarose slurry was added to the lysates and incubated for 2 hours at 4 °C. For Western blot analysis, proteins were separated by SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore). After protein transfer, the membranes were treated with primary antibody for 3 hours at room temperature and followed by incubation with HRP-conjugated goat anti-mouse antibody. After washing the membranes three times and each for ten minutes, signals were visualized using Amersham ECL Prime Western Blotting Detection Reagent (GE healthcare). For immunoprecipitation experiments in Fig 3, 0.2 μg of mC* construct was used to minimize PKAc transgene expression. For GST in vitro binding assays, GST-Smo fusion proteins were produced in *E. coli* and purified with Glutathione-agarose beads (GE Healthcare). GST fusion proteins bound to the glutathione beads were washed three times with ice-cold PBS containing 1% NP-40, and were incubated with a recombinant PKAc (500 U for each mix; New England Biolabs) for 1 h at 4°C with occasional mixing. The beads were washed again three times with PBS plus 1% NP-40 before separation on SDS-PAGE, followed by Western blot using a PKAc antibody (sc-903, Santa Cruz). Antibodies used for this study are Ci [2A1 (85)], Ptc (Apa1, DSHB), En (4D9, DSHB), HA (sc-7392, Santa Cruz), LacZ (G8021, Sigma), GFP (G-10362, Invitrogen), Flag (F-3165, Sigma), PKAc (sc-903, Santa Cruz).

Luciferase assay and RNAi in cultured *Drosophila* cells

dsRNAs were generated using the MEGAscript High Yield Transcription Kit (Ambion). The following primers were used for generating the dsRNA targeting *smo* 5' UTR: 5'-GAATTAATACGACTCACTATAGGGAGAGT CGCACATTTGTTGCTTCAG-3' and 5'-GAATTAATACGACTCACTATAGGGAGACCGCTTA TAAAAATCATTAATA-3'. dsRNA targeting the coding sequence of firefly luciferase was used as a negative control. For knockdown experiments, S2 or C18 cells were cultured in serum-free medium containing *Smo* or control dsRNA for 8 hours at 24°C. FBS was added to a final concentration of 10% for S2 cells or 2.5% for C18 cells and cells were cultured for 24 hours before transfection with the following plasmids: 1 µg *ptc-luc* reporter construct (86), 50ng RL-PolIII renilla construct (87) and 1 µg of the different *Smo* constructs in 12 well plates. After 48 hours, cells were lysed and the reporter assays were performed using the Dual-Luciferase reporter assay system (Promega), measured in triplicate using a FLUOstar OPTIMA plate reader (BMG LABTECH, Germany).

FRET analysis

FRET analysis was carried out as previously described (15). CFP- and YFP-tagged constructs were transfected into S2 cells together with the *ubi-Gal4* expression vector (29). Cells were washed with PBS, fixed with 4% formaldehyde for 20 minutes, and mounted on slides in 80% glycerol. The intensity of CFP emission was acquired with the 100X objective of Zeiss LSM510 confocal microscope before (BP) and after (AP) photobleaching of YFP with 514 nm laser at full strength for 100 seconds. Each data point was calculated using 10 to 20 individual cells. In each cell, four or five regions of interest in the photobleached area were selected for analysis. The average intensity of CFP emission for each region was quantified by ImageJ software and regions for each cell were averaged. FRET efficiency was calculated as: $\text{FRET \%} = [(CFP_{AP} - CFP_{BP}) / CFP_{AP}] \times 100$. For FRET analyses in wing discs, CFP- and YFP-tagged *UAS* transgenes were expressed using *MS1096*. CFP signals were acquired with 63X objective of Zeiss LSM510 confocal microscope before (BP) and after (AP) photobleaching YFP from 3 regions of interest per disc. Images were processed, and FRET % calculated as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1.

Hh signaling stabilizes a constitutively active form of PKAc in wing imaginal discs. (A-C, E, and F) Images of late third instar wing imaginal discs immunostained with PKAc (red) and En (green) antibodies. En expression marks P compartment cells and A compartment cells adjacent the A/P boundary. Wing discs expressed *UAS-mC** (A) or coexpressed *UAS-mC** and *UAS-Smo-Fg* (B), *UAS-Smo-Fg* and *UAS-Hh* (C), *UAS-SmoSA-Fg* (E), or *UAS-SmoSD-Fg* (F) using *ap-Gal4*. (D) Images of late third instar wing discs coexpressing *UAS-mC** with *UAS-Smo-Fg* using *MS1096* and containing *ptc^{IIW}* mutant clones (marked by the lack of GFP). The discs were immunostained with PKAc (red) and GFP (green) antibodies. Expression of *UAS-Hh* (C) or *UAS-SmoSD* (F), or *ptc^{IIW}* mutant clones (D) caused wing

disc overgrowth, whereas expression of *UAS-SmoSA* (E) inhibited disc growth. Wing discs are oriented with anterior toward left and ventral up. Images are representative of N = 5 wing discs per genotype.

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Fig 2. Hh signaling increases the activity of PKA at the plasma membrane. (A) Graph of the FRET efficiency from AKAR3 in S2 cells expressing mC* or stimulated with Hh or forskolin. (B) Subcellular localization of AKAR3 and Myr-AKAR3 in S2 cells. (C-H) Graph of the FRET efficiency from Myr-AKAR3 in S2 cells (C-E) expressing mC* or stimulated with Hh or forskolin (C), expressing Myr-mC* or Myr-KD (kinase dead PKAc) (D), transfected with negative control or Smo dsRNAs and stimulated with Hh (E), or in late third instar wild type wing discs (F-H) containing *ptc*^{IIW} (G), or *smo*³ (H) mutant clones. For A, C-E, data are means $\pm$ S.D. of 10 cells from 3 biological replicates. For F-H, data are means $\pm$ S.D of 10 discs (3 fields of interest per disc) for each genotype. * $P < 0.05$, ** $P < 0.01$ Student's *t*-test.

Fig 3.

Hh induces the formation of a Smo-PKAc complex formation that stabilizes PKAc. (A-C) Late third instar wing imaginal discs coexpressing *UAS-mC** and *UAS-GFP-Smo* using *MS1096* immunostained for PKAc (red) and GFP (green). High magnification views of the posterior compartment (B) and anterior compartment (C) are shown. Images are representative of N = 5 (A) or 10 (B-C) wing discs. (D-E) Western blots of coimmunoprecipitation experiments from lysates of S2 cells transfected with the indicated constructs. Cells were grown in the presence or absence of Hh-stimulation for 24 hours and exposed to MG132 (50 μ M) for 4 hours prior to harvest. Cells in E were also exposed to forskolin (7.5 μ M) for 1 hour prior to harvest. The antibody against GFP was used to detect mC*-YFP. (F-H) Western blots of lysates from S2 cells that were transfected with mC*-YFP and Myc-Smo, Myc-SmoSA, or Myc-SmoSD, and grown in the absence or presence of Hh-conditioned medium for 24 hours and cyclohexamide (CHX, 100 μ M) for the indicated times. Myc-CFP was cotransfected as an internal control. Western blots are representative of 3 independent experiments (D-H). Graphs indicate the quantification of the intensity of mC*-YFP calculated as a percent of that at time = 0. Data are means $\pm$ S.D. from 3 biological replicates.

Fig 4. Multiple clusters of basic amino acids mediate Hh-induced binding of PKAc to Smo. (A) S2 cells coexpressing mC*-YFP and C-terminally CFP-tagged wild type (SmoWT) or the indicated Smo deletion mutants. Images are representative of N = 10 cells from 3 biological replicates. In the drawings on the left, the blue bars indicate the transmembrane helices. (B) Drawing of wild-type Smo and the sequences of three clusters of basic amino acids (R1', R2' and R3') in the juxtamembrane region of the C-tail of Smo. Amino acid substitutions for individual RA mutations are depicted below. The red box indicates and the SAID domain. (C) Western blots of in vitro binding assays using purified GST-Smo fusion proteins containing the indicated Smo C-tail fragments and recombinant PKAc. (D-E) S2 cells transfected with mC*-YFP and the indicated CFP-tagged Smo deletion variants, grown in the absence or presence of Hh-conditioned medium. Representative confocal micrographs (D) and graph of FRET efficiency (E). Images are representative of N = 10 cells from 3

biological replicates (D). FRET data are means $\pm$ S.D. N=10 cells from 3 biological replicates (E). $**P<0.01$ Student's *t*-test.

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Fig 5.

Inhibition of Hh-induced binding of PKAc to Smo blocks trans-phosphorylation of Smo and activation of Hh signaling. (A) Western blot of coimmunoprecipitates from lysates of S2 cells transfected with the indicated constructs. Cells were unstimulated or Hh-stimulated for 24hours and exposed to MG132 (50 μ M) for 4 hours and forskolin (7.5 μ M) for 1 hour prior to harvest. (B, D) S2 cells were transfected with the indicated Smo constructs in the absence (B) or presence (D) of Smo dsRNA, treated with or without Hh-conditioned medium, followed by immunoprecipitation and Western blot analysis with the indicated antibodies. (C, E) *ptc-luc* reporter assays in C18 cells transfected with the indicated Smo constructs in the absence or presence of Hh-conditioned medium and dsRNA that targets the 5' UTR of the endogenous *smo* mRNA (SmoRNAi 5'UTR). Data are means $\pm$ S. D. of normalized luc activity from 3 independent experiments. (F) S2 cells were transfected with the indicated constructs in the absence or presence Hh-conditioned medium, followed by immunoprecipitation and western blot analysis with the indicated antibodies. (G) S2 cells were transfected the indicated Smo constructs in the presence of Smo dsRNA, treated with or without Hh-conditioned medium, followed by immunoprecipitation and western blot

analysis with the indicated antibodies. For A-B, D, and F-G, western blots are representatives of 3 independent experiments.

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Fig 6.

PKAc-binding deficient forms of Smo are defective in transducing Hh signal in wing imaginal discs. (A-B'') Images of late third instar wing imaginal discs containing *smd3* mutant clones induced by FRT/FLP-mediated mitotic recombination were immunostained to show the expression of GFP, Ci, Ptc and En. *smd3* mutant clones are marked by GFP (arrows). (C-H'') Late third instar wing discs containing *smd3* mutant clones also expressing *tub>Myc-Smo* (C-D''), *tub>Myc-SmoRA2'3'* (E-F'') or *tub>Myc-SmoRA1'2'3'* (G-H'') were immunostained to show the expression of Myc, Ci, Ptc and En. *smd3* clones expressing Smo transgenes are marked by Myc expression (arrows). (I-J'') Late third instar wing discs containing *smd3* mutant clones also expressing *C765>Myc-SmoRA2'3'* (I-I'') or *C765>Myc-SmoRA1'2'3'* (J-J'') were immunostained to show the expression of Myc, Ci and Ptc. Images are representatives of N = 5 wing discs per genotype.

Fig 7.

Hh regulates the formation of distinct PKAc-substrate complexes. (A) S2 cells were cotransfected with CFP-tagged Ci^{PKA}, YFP-tagged mC*, Flag-tagged Cos2 and Myc-tagged Smo and treated with or without Hh-conditioned medium. Cells were immunostained with Flag or Myc antibody to visualize Flag-Cos2 or Myc-Smo, respectively. Ci^{PKA}-CFP and mC*-YFP were visualized by CFP and YFP fluorescence, respectively. Images are representatives of N = 10 cells from 3 biological replicates. (B) FRET efficiency between Cos2-CFP and mC*-YFP, between Smo-CFP^{L3} and mC*-YFP, or between Ci^{PKA}-CFP and mC*-YFP. S2 cells were transfected with Cos2-CFP and mC*-YFP, Smo-CFP^{L3} and mC*-YFP, or Ci^{PKA}-CFP and mC*-YFP in the presence of absence of Myc-Smo or/and Flag-Cos2, treated with or without Hh-conditioned medium, followed by FRET analysis using confocal microscopy. FRET data are means $\pm$ S.D. N=10 cells from 3 biological replicates. **P<0.01 student's t-test. (C) A model for Hh-regulated formation of distinct PKAc-substrate complexes. In the absence of Hh, PKAc forms an intracellular complex with Cos2 and Ci to phosphorylate Ci, targeting it for SCF^{Slimb}-mediated proteolytic processing to generate Ci^R. Smo is internalized and degraded. In the presence of Hh, PKAc forms a

complex with Smo to phosphorylate Smo C-tail, likely by engaging in phosphorylation both *in cis* (blue arrow) and *in trans* (red arrow). Phosphorylation of Smo C-tail induces its conformational change and oligomerization, leading to Fu activation. Activated Fu blocks Ci processing into repressor form (Ci^R) and converts full-length Ci into labile activator form (Ci^A). Green lines denote the PKAc binding region in Smo C-tail.