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Hedgehog (Hh) signaling plays a key role in the development and maintenance of animal tissues. This signaling is mediated by the atypical G protein–coupled receptor (GPCR) Smoothened (Smo). Smo activation leads to signaling through several well-characterized effectors to activate Hh target gene expression. Recent studies have implicated activation of the heterotrimeric G protein subunit Ga, and the subsequent decrease in cellular cAMP levels in promoting the Hh response in flies and mammals. Although Hh stimulation decreases cAMP levels in some insect cell lines, here using a bioluminescence resonance energy transfer (BRET)-based assay we found that this stimulation had no detectable effect in Drosophila S2-R+ cells. However, we observed an unexpected and significant Ga, dependent increase in cAMP levels in response to secreted Hedgehog (Hh) family proteins. Insufficient or excessive Smo activity can have severe consequences for the formation and maintenance of tissues (1, 2). In fact, activating mutations in Smo have been shown to drive tumor formation in several tissues (1, 3). This suggests that Smo activity must be tightly regulated in cells. Consistent with this, several complex and intersecting regulatory mechanisms for finely controlling Smo activity have been identified. Many of the details are best understood in Drosophila melanogaster, where ubiquitination (4, 5), sumoylation (6, 7), and phosphorylation (8–13) of the Smo cytoplasmic C-terminus (SmoCT) all play key roles in controlling Smo protein levels, localization, and activity.

SmoCT phosphorylation seems to be a particularly important means of controlling Smo activity. Phosphorylation of the Smo autoinhibitory domain (SAID) region of the SmoCT by the cAMP-regulated protein kinase A (PKA) is the principal mechanism for activating Drosophila Smo. PKA phosphorylation primes the SAID for phosphorylation at adjacent sites by CkI, and the combined effects are sufficient to stabilize Smo, promote its accumulation at the plasma membrane, and drive it into an active conformation (8–10, 14). However, other kinases, including GPCR kinase 2 (Gprk2)(11), CkIγ(12), and Fu (13), act after PKA to modulate Drosophila Smo activity by phosphorylating other sites in the SmoCT. Like the PKA phosphorylation sites, most of these sites are not conserved between flies and vertebrates. Interestingly, however, Gprk2 and a homologous mammalian kinase, GRK2 (together with casein kinase I), phosphorylate conserved sites in the proximal SmoCT in Drosophila and mice, respectively (11, 15). In both organisms, phosphorylation of these sites promotes Smo signaling activity (11, 15, 16), indicating that GRK phosphorylation of the SmoCT is an evolutionarily conserved mechanism for Smo activation (although it may not be critical in all species) (17). However, precisely how GRK phosphorylation enhances Smo activity is not known.

Once activated, Smo signals through a series of downstream canonical effectors that include the atypical kinesin Costal-2/
The role of PKA in the Hh response has been controversial. Experiments using a cAMP-insensitive form of the PKA catalytic subunit initially shed doubt upon the importance of regulated PKA activity in the Hh response (31, 32). However, several studies cited above have now shown clear links between Goαi proteins, cAMP, and Hh pathway activity. The link between decreased cAMP production and increased Hh target gene expression seems fairly straightforward in mammals, where the principal role of PKA is to inhibit GLI proteins (2). However, there is conflicting evidence showing that PKA can also promote Shh signaling in vertebrate cells and tissues (33, 34). The situation is similarly complex in Drosophila, where PKA functions both as an activator and inhibitor of the Hh response. For example, complete loss of PKA activity causes constitutive Ci activation and ligand-independent target gene expression (31, 35), but more moderate PKA inhibition or reduction of cAMP levels can actually decrease Hh-dependent target gene expression (8, 36). Similarly, increased cAMP levels or PKA activity can either prevent (31, 37) or enhance (8, 36, 38) expression of Hh target genes. The evidence suggests that control of cAMP levels within certain upper and lower limits is important for proper functioning of the pathway. Some of the conflicting evidence may result from cell-type–specific effects that could reflect differences in basal cellular cAMP levels in different systems (39).

To address some of the conflicting evidence about the implication of heterotrimeric G proteins in the Hh response and to examine how G protein signaling could be regulated at the level of Smo, we sought to characterize the effects of Hh signaling on cAMP levels and target gene expression using cell-based assays in Drosophila S2 cells. To our surprise, we did not observe changes in cAMP levels in Hh-treated cells. However, stronger activation of Smo caused a substantial Goαs-dependent increase in cAMP levels specifically in response to Gprk2 phosphorylation of the SmoCT. This effect on cAMP production was mediated by the evolutionarily conserved core of Smo, and occurred independently of the canonical signaling effectors Fu and Cos2. Although not essential for signaling, Goαi and Goαs depletion experiments in vivo both suggest that heterotrimeric G protein-mediated regulation of cAMP levels is important for adjusting the sensitivity of cells to endogenous Hh ligand levels.

Results

Smo can activate Goαs-dependent signaling

Drosophila Smo has been shown to activate Goαs in transfected S9 cells and Hh–treated Clone-8 cells (21, 40). Initially to see if we could observe the same response, we used a bioluminescence resonance energy transfer (BRET) assay based on the EPAC-BRET biosensor (41) to measure changes in cAMP levels in live S2 cells. The BRET signal produced by this biosensor is inversely correlated to cAMP levels. In control experiments, stimulation of cAMP production by expression of a partially activated mutant form of Goαs (GoαsQ215L) (42) caused the expected decrease in the EPAC-BRET signal (Fig. 1A). Conversely, inhibiting cAMP production with the comparable activated form of Goαs (GoαsQ206L) significantly increased the EPAC-BRET signal (Fig. 1A). Thus this assay can reliably detect both increases and decreases in cellular cAMP levels in S2 cells.

We then conducted EPAC-BRET assays to test for Hh-dependent changes in cellular cAMP levels. Transfection of S2 cells with an expression plasmid for HhN, a secreted and biologically active N-terminal fragment of Hh (43) did not yield the expected decrease in cellular cAMP levels, although it did activate target gene expression (assessed using a ptc-luciferase reporter assay) (44) (Fig. 1B and C). Even when using a myristoylated, membrane-targeted form of the EPAC-BRET biosensor to more specifically assess changes in cAMP pools at the plasma membrane (45), we did not observe the anticipated decrease (Fig. S1).

To see if enhancing Smo activity would lead to an effect in this assay, we expressed GFP-tagged, WT and mutant forms of Smo (see Fig. 1D), ptc-luc reporter activity was similarly low in WT Smo (SmoWT)-transfected cells compared with mock transfected controls (Fig. 1E), indicating that the level of Smo overexpression was moderate. Co-expression with HhN caused strong activation of ptc-luc reporter transcription (Fig. 1E). Unexpectedly, we observed a substantial Hh-dependent increase in cellular cAMP levels under these conditions (Fig. 1F). We obtained the same effect when we strongly activated signaling by expressing the PKA- and Cki-phosphomimetic,
constitutively active mutant form of Smo, SmoSD (hereafter referred to as SmoSD) (8) (Fig. 1, G and H). Thus strong activation of the Hh pathway increased both target gene expression and cAMP levels in S2 cells.

To determine whether the increase in cAMP levels we observed is an acute response to Smo activation rather than a gradual or adaptive response to days-long pathway activation in our HhN co-transfection experimental setup, we carried out a time course analysis on SmoWT-expressing cells treated with medium conditioned by control- or HhN-expressing cells. Treatment with control-conditioned medium had no effect on cAMP levels at any time point tested (Fig. 1). In short-term treatments (between 1 and 10 min), we did not observe HhN-dependent changes in cAMP levels (results not shown). Increases in cAMP levels were observed after 30 and 60 min of exposure to HhN, but they did not reach statistical significance (Fig. 1). After 2 h of HhN treatment, cAMP levels were significantly increased, and by 4 h the magnitude of increase was similar to that observed in control cells that had expressed co-transfected HhN for 2–3 days (Fig. 1). In Western blot analysis at each of these time points, HhN-dependent stabilization of Smo-GFP was just detectable after 30 min of treatment, and the shifted, hyperphosphorylated active form of Smo-GFP first became readily apparent after 2 h of HhN exposure and increased at 4 h (Fig. 1). Thus the time course of cAMP regulation closely matched the profile of Smo activation. Although

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Figure 1. Strong Smo activation stimulates cAMP production in S2 cells. Graphed data represent mean ± S.D. A, EPAC-BRET assay to measure changes in cAMP levels in S2-R+ cells in response to expression of constitutively active forms of Gs (GαsQ215L) or Gi (Gιαi205L), t test versus cells transfected with empty vector (-); **, p < 0.01; ***, p < 0.001. B, ptc-luc reporter assay monitoring target gene activation in mock- and HhN-transfected S2-R+ cells. t test: ***, p < 0.001.

C, measurement of cAMP levels by EPAC-BRET assay in mock- and HhN-transfected S2-R+ cells. t test: n.s., not significant. D, schematic diagram of truncated/mutated Smo variants used in this study (not to scale). The relative locations of the 7-transmembrane domains (black boxes), the four clusters of Gprk2 phosphorylation sites (green boxes), and the SAID containing three clusters of PKA/Cki phosphorylation sites (blue box) are indicated. Numbers refer to amino acid positions in Smo. E, ptc-luc reporter assay monitoring target gene activation in control and SmoWT-GFP transfected cells, with and without HhN co-transfection. t test: ***, p < 0.001. F, measurement of cAMP levels by EPAC-BRET assay in cells transfected with empty vector (-), SmoWT-GFP, or SmoWT-GFP plus HhN. t test: ***, p < 0.001; n.s., not significant. G, ptc-luc reporter activity in cells transfected with empty vector (-) or SmoSD-GFP. H, EPAC-BRET assay of cAMP levels in SmoWT-GFP transfected cells treated for 0.5, 1, 2, or 4 h with medium conditioned by control cells or cells expressing HhN, or co-transfected with HhN expression plasmid (Smo + Hh). t test: ***, p < 0.001; n.s., not significant. J, immunoblot (IB) analysis of SmoWT-GFP in cells treated as in I.
GRK-phosphorylated Drosophila Smo activates $\Gamma_s$ signaling

Figure 2. The increase in cAMP downstream of Smo is $\Gamma_s$-dependent. Graphed data represent mean ± S.D. A, immunoblot (IB) analysis of myc-tagged Smo$\alpha$-transfected S2-R+ cells, treated with dsRNAs targeting β-gal (−) or Gas. The blot was probed with anti-myc tag antibody to show efficient depletion of transfected Smo$\alpha$, and with anti-α-tubulin as a loading control. B, EPAC-BRET assay of cAMP levels in control (−) or Smo$\alpha$-GFP-transfected S2-R+ cells treated with dsRNA targeting β-gal (control) or Gas. t test: ***; $p < 0.001$; n.s., not significant. C, immunoblot analysis of cells treated as in B. Blot was probed with anti-GFP antibody to reveal Smo$\alpha$-GFP expression and anti-α-tubulin as a loading control.

the cAMP increase was similar in cells expressing HhN for days and those treated for only 4 h, the transiently exposed cells had only a small percentage of the levels of Smo protein observed in the continuously exposed cells (Fig. 1J). This suggests that the effect does not require extremely high levels of Smo protein.

The most straightforward interpretation of these results is that strong activation of Smo activates $\Gamma_s$, either directly or indirectly (e.g. by regulating another GPCR). If this is true, we expected that double-stranded RNA (dsRNA)-mediated depletion of $\Gamma_s$ would block the increase in cAMP levels. We first confirmed that treatment of $\Gamma_s$-transfected cells with dsRNA targeting the gas transcript efficiently blocked production of the protein (Fig. 2A). Consistent with our expectation, the ability of Smo to increase cAMP levels was strictly dependent upon $\Gamma_s$. dsRNA-mediated depletion of $\Gamma_s$ had no significant effect on basal cAMP levels in control cells (Fig. 2B). However, the Smo$\alpha$-dependent increase in cAMP levels was almost completely inhibited (Fig. 2B). We confirmed that Smo$\alpha$ protein levels were unchanged by $\Gamma_s$ depletion (Fig. 2C). We conclude that strong activation of Smo activates $\Gamma_s$, either directly or indirectly, to stimulate cAMP production in S2 cells.

GRK phosphorylation of SmoCT triggers $\Gamma_s$-dependent downstream signaling

Although PKA/CkI phosphorylation is the principal trigger for Smo activation, more than half of the transcriptional response observed in ptc-luc reporter assays depends upon phosphorylation of Smo by Gprk2 (11). To see if Gprk2 affects heterotrimeric G protein–dependent signaling downstream of Smo, we treated S2 cells with a gprk2-specific dsRNA. The ability of Smo to activate $\Gamma_s$ depended upon Gprk2, as efficient depletion of the kinase strongly impaired the ability of Smo$\alpha$ to stimulate cAMP production (Fig. 3, A and B). To confirm that this is due to a direct effect of Gprk2 phosphorylation on Smo activity, and not to an indirect effect of Gprk2 on CAMP regulation (as described previously in Ref. 36), we targeted the Gprk2 phosphorylation sites in SmoCT rather than the kinase. Consistent with the depletion experiments, mutating the four clusters of Gprk2 phosphorylation sites to Ala (in Smo$\alpha$c1–4A) prevented Smo$\alpha$ from stimulating cAMP production (Fig. 3C). However, Smo$\alpha$c1–4A was well-expressed and retained substantial ability to stimulate target gene expression (~20-fold over baseline levels) (Fig. 3, D and E). Although phosphorylation by PKA and CkI is sufficient to stabilize Smo and activate some signaling, we conclude that it is phosphorylation by Gprk2 that specifically triggers $\Gamma_s$-dependent downstream regulation of cAMP levels.

$\Gamma_s$-dependent signaling is mediated via the conserved core of Smo

We previously showed that a C-terminally–truncated form of Smo that retains only the broadly conserved core of the protein (amino acids 1–663; Smo$\alpha$core) is constitutively active and capable of activating Hh target gene expression, although less effectively than Smo$\alpha$ (Fig. 4A) (11). This suggested that Smo proteins from different species share common aspects in their signaling mechanisms (2, 46). We found that Smo$\alpha$core possesses all the sequences necessary to promote $\Gamma_s$-dependent signaling, in a constitutive manner. In Smo$\alpha$-expressing cells, cAMP levels increased to a similar extent as in Smo$\alpha$-expressing cells (Fig. 4, B and C), both throughout cells and more specifically at the membrane (Fig. S1), and in a $\Gamma_s$-dependent manner (Fig. 4, D and E). In Smo$\alpha$-expressing cells, co-expression of Ptc almost completely suppressed both ptc-luc reporter activation and the increase in cAMP production (Fig. 4, A–C). This suggests that the stimulation of cAMP production is a specific, Hh-regulatable signaling activity of Smo$\alpha$core.

We tested additional mutants to identify the determinants in Smo$\alpha$core required to activate $\Gamma_s$-dependent signaling. Unlike full-length Smo, Smo$\alpha$core activity is entirely dependent upon Gprk2 phosphorylation (Fig. 4F) (11). Its ability to drive $\Gamma_s$-dependent signaling was similarly reliant upon Gprk2 phosphorylation, as stimulation of cAMP production by Smo$\alpha$core was strongly impaired by either depletion of Gprk2 (Fig. 4, G and H) or mutation of the Gprk2 phosphorylation sites (Smo$\alpha$corec1–3A) (Fig. 4, I and J). We previously showed that the C-terminus of Smo$\alpha$core (between amino acid 626 and 663) is required for its activity in ptc-luc reporter assays (11). This same region is also required for $\Gamma_s$ activation (Fig. 4, K–L). Thus the 7-transmembrane domain region of Smo is not sufficient to activate $\Gamma_s$-dependent signaling; sequences in the broadly-conserved membrane-proximal portion of the cytoplasmic tail are also required.

Activation of $\Gamma_s$-dependent signaling downstream of Smo is distinct from HSC signaling

Phosphorylation of Smo by Gprk2 affects canonical signaling through the HSC in complex ways. For example, it promotes Smo C-terminal dimerization, which itself promotes Fu activa-
GRK-phosphorylated Drosophila Smo activates G\(\alpha_s\) signaling

Figure 3. Gprk2 phosphorylation of the SmoCT activates G\(\alpha_s\)-dependent signaling. Graphed data represent mean ± S.D. t test: ***, \(p < 0.001\). A, EPAC-BRET assay measuring cAMP levels in mock (−) or Smo\(^{SD}\)-transfected S2-R+ cells treated with dsRNA targeting β-gal (control) or gprk2. Blot was probed with anti-GFP antibody to reveal Smo\(^{SD}\)-GFP expression, anti-Gprk2 to show efficient depletion of Gprk2 protein, and anti-α-tubulin as a loading control. B, C, and D, EPAC-BRET assay measuring cAMP levels (C) and ptc-luc reporter assay (D) in control (−), Smo\(^{SD}\)-GFP, and Smo\(^{SDc1–4A}\)-GFP-transfected cells. E, immunoblot analysis of cells treated as in C. Blot was probed with anti-GFP antibody to reveal Smo\(^{SD}\)-GFP expression and anti-α-tubulin as a loading control.

Activation of signaling through the HSC depends upon interaction of activated Smo with Cos2 (50, 51). To test if Cos2 also directly participates in \(G\alpha_s\)-dependent signaling downstream of Smo, we made use of a dominant-negative form of Cos2 consisting of just the cargo domain, termed the C-terminal Smo-binding domain (CSBD). This protein targets the interaction between Smo and Cos2 and thereby attenuates Hh signaling (40). As expected, CSBD expression almost completely inhibited ptc-luc reporter activation downstream of Smo\(^{SD}\) (Fig. 5G), and this was associated with a strong reduction in Smo protein levels (as others have seen) (40) (Fig. 5H). Interestingly, however, CSBD had no significant effect on the ability of Smo\(^{SD}\) to stimulate cAMP production despite the decrease in Smo\(^{SD}\) protein levels (Fig. 5J). This suggests that the pool of Smo that signals to \(G\alpha_s\) may be different from the Cos2-associated pool signaling through the HSC. Together, these results suggest that \(G\alpha_s\)-dependent signaling downstream of Smo is separable and distinct from signaling through the HSC components Cos2 and Fu.

Our analysis to this point suggested that \(G\alpha_s\) may act in parallel to the HSC downstream of Smo, with both cooperating to promote Hh target gene expression. If so, then the loss of \(G\alpha_s\)-mediated signaling might explain the decrease in target gene expression we observe when Gprk2 phosphorylation of Smo is blocked. If this were true, then increasing cAMP levels should rescue target gene expression downstream of the Gprk2 non-phosphorylatable Smo variant. We used the activated \(G\alpha_s^{Q215L}\) mutant to test this. Expression of either \(G\alpha_s^{Q215L}\) or Smo\(^{SD}\) increased cellular cAMP levels (Fig. 5F), but only Smo\(^{SD}\) drove ptc-reporter expression as expected (Fig. 5K). Both effects depended upon Gprk2 phosphorylation of the SmoCT, as expected (Fig. 5, J and K). Although \(G\alpha_s^{Q215L}\) restored the increase in cAMP levels in Smo\(^{SDc1–4A}\)-expressing cells (Fig. 5F), it did not rescue ptc-reporter activation (Fig. 5K). These results suggest that phosphorylation of Smo by Gprk2 does something else to downstream signaling besides promoting \(G\alpha_s\) activity.

\(G\alpha_s\) enhances the response to low levels of Hh

Having not seen any obvious role for heterotrimeric G protein signaling in the endogenous response of S2 cells to Hh\(^N\)-conditioned medium, we turned to an \textit{in vivo} system to see if \(G\alpha_s\) plays any physiological role in Hh signaling. First, we generated clones of cells homozygous for a null allele of \textit{gsp} (\textit{gsp}\(^{R60}\)) in wing discs using the FLP-FRT system (52). These clones were small when generated in an otherwise WT background. Even when generating them \textit{in vivo} in a Minute heterozygous background to give them a growth advantage (53), \textit{gsp}\(^{R60}\) clones were only about half the size of WT clones generated in parallel (Fig. 6, A−C). We did not observe changes in intermediate or high-threshold Hh responses (expression of Ptc and Engrailed, respectively) in \textit{gsp}\(^{R60}\) clones at the anterior/posterior (A/P) boundary (not shown). However, we did observe an effect on expression of the low threshold target gene \textit{dpp} (assessed using a \textit{dpp}-LacZ enhancer trap line). Normally, \textit{dpp}-LacZ staining is fairly symmetrical in dorsal and ventral compartments of the wing pouch (see below) (Fig. 6D). However, in some larger \textit{gsp}\(^{R60}\) clones located away from the A/P boundary, \textit{dpp}-LacZ
expression was reduced (Fig. 6, E and F). As the concentration of Hh is thought to decline with increased distance from the P compartment where it is produced, this suggests that Gαs may play a role in the normal response to Hh where ligand levels are low.

We were concerned that this approach might underestimate the role of Gαs because of the potential for nonautonomous rescue in mosaic discs. Wing discs cells have been demonstrated to be highly connected through gap junctions, with small dyes able to diffuse over many cell diameters at late third instar stage (54). cAMP has a lower molecular weight than these dyes, suggesting that it too may be able to diffuse in discs. Diffusion of cAMP from neighboring WT cells might limit the cell-autonomous effects in gαs R60 clones.

To circumvent this potential complication, we used expression of a dsRNA transgene to deplete Gαs throughout a much larger territory in the disc. Expression of Gαs dsRNA throughout the developing wing pouch (using nubbin-GAL4) caused a failure in wing inflation (Fig. 7, A and B), a phenotype that has been linked to a failure in Gαs activation downstream of the GPCR Rickets (55). This suggests that the dsRNA transgene does in fact deplete Gαs.

Consistent with the clonal analysis, depletion of Gαs selectively impaired low threshold dpp expression. We used apterous (ap)-GALA4 to drive expression of Gαs dsRNA throughout the dorsal compartment of the wing disc, leaving the ventral compartment as an internal WT control. In control ap-GALA4/+ wing discs, low threshold Hh responses (such
as stabilization of full-length Ci (Ci155) and expression of dpp
and intermediate threshold responses (expression of Ptc) appeared similar in the GAL4-expressing dorsal and WT ventral compartments (Fig. 7E). Quantification and averaging of the relative fluorescence intensities from several discs confirmed that both compartments show virtually identical Hh responses both in terms of intensity and distance from the A/P boundary (Fig. 7F).

ap-GAL4-driven expression of Gαs dsRNA had little effect on Ci stabilization or Ptc expression (Fig. 7, G and H). The most striking effect was a strong and statistically significant reduction in dpp expression (Fig. 7, G and H). Most notably, the A-P distance over which dpp was expressed was substantially less in the Gαs-depleted dorsal compartment, indicating that cells exposed to low levels of Hh were no longer able to mount an effective transcriptional response. This fits well with our observations of gαsΔ60 mutant clones, and suggests that Gαs plays a positive signaling role in situations where Hh levels are low and limiting for a response.

Consistent with this interpretation, we observed a Hh concentration-dependent requirement for Gαs in Smo-transfected S2 cells. In a HhN dose-response test, we found that the standard conditions we used for testing Hh induction of the pathway (100 ng of transfected HhN plasmid) produced approximately a 60% maximal response in ptc-luc reporter assays (Fig. 8A). Under these conditions, we did not observe a significant effect of Gαs depletion on ptc-luc reporter expression (Fig. 8B). However, at a lower level of Hh (20 ng of HhN plasmid), which was sufficient for ~25% maximal response in ptc reporter assays (Fig. 8A) and for stimulating cAMP production (Fig. 8C), depletion of Gαs decreased Hh-dependent ptc-luc reporter activation by Smo-GFP (Fig. 8D). The effect was modest (~25% decrease), suggesting that signaling through Gαs is not essential
for the response. Rather, this result together with our in vivo analysis suggest that Gαi enhances responsiveness to low levels of Hh in vivo.

**Gαi depletion reveals an inhibitory role for this protein in the Hh response**

Our analysis of Gαi function appears to contradict previous studies in several systems showing that inhibition (rather than stimulation) of cAMP production, through Gαi, promotes Hh target gene expression (21, 22). In particular, gai mutant clones in wing discs, which appeared fairly small, failed to express dpp (22). We tested whether depletion of Gαi over a larger territory would produce a similar effect. Expression of a dsRNA targeting Gαi throughout the developing wing pouch (using the nubbin-GAL4 driver) impaired growth, causing the resulting adult wings to be undergrown, similar to those of gai hypomorphic mutants (Fig. 7C) (40). This suggests that the dsRNA did target Gαi. Interestingly, the wings were not uniformly smaller. Instead, the size of the central region bounded by longitudinal wing veins 3 and 4 (L3 and L4) as a proportion of total wing area was significantly increased upon Gαi depletion (Fig. 7D). This is a characteristic feature of increased Hh signaling in the wing disc. Consistent with this interpretation, Gαi depletion throughout the dorsal compartment significantly increased the level and extent of dorsal CI155 stabilization and expression of dpp and Ptc (Fig. 7I and J). Compared with control discs, cells further from the A/P boundary (and thus exposed to lower levels of ligand) in the Gαi-depleted discs were able to activate robust target gene transcription. This suggests that Gαi depletion enhanced sensitivity of cells to low levels of Hh, which is the opposite of the effect of Gαs depletion. Our analyses of Gαi and of Gαs function in wing discs are thus consistent in revealing a positive effect of cAMP on sensitivity to Hh in vivo.

**Discussion**

Despite conflicting evidence about the ability and necessity of Smo itself activating Gαi signaling, heterotrimeric G proteins have increasingly been linked to Hh signaling under normal and pathological circumstances (e.g.Refs. 29 and 56). The emerging model (57), based largely on observations in mammalian cells, suggests that cAMP plays primarily an inhibitory role in the pathway. In the absence of Hh, a Gαi-coupled GPCR (like Gpr161 in mammals) signals to maintain cAMP at high levels, thereby ensuring efficient PKA phosphorylation and processing of GLI proteins to their repressor forms. In response to Hh ligands, decreased Gαi signaling and/or increased Gαs signaling by Smo or certain other Gαi-coupled GPCRs lowers cAMP levels, allowing GLI proteins to avoid PKA phosphorylation and accumulate in their full-length activator forms. However, the situation must be more complex, as CAMP/PKA cannot only inhibit but also promote Hh signaling in both flies and mammals (8, 33, 34, 36, 38), most directly in the case of *Drosophila* by phosphorylating and activating Smo. Consistent with this, we have observed a Gαs-dependent increase in cellular CAMP levels downstream of Smo activation, and identified a positive role for Gαs in Hh signaling in vivo.

We expected to see Gαi activation and a decrease in CAMP levels in Hh-treated S2 cells, similar to what was previously observed in other insect cell lines (21, 22). However, we observed no consistent effect on total CAMP levels in S2 cells exposed to HhN. Studies in mouse cerebellar granule cell progenitors suggested that Hh signaling is controlled by a relatively small pool of cAMP that is restricted in its localization, likely near the cilium (58). Hh-dependent changes in CAMP levels may similarly be restricted to a smaller pool in S2 cells such that they do not significantly impact the total CAMP pool; in fact, using a membrane-anchored, myristoylated FRET biosensor of PKA activity in S2 cells, Li et al. (45) observed an ~2-fold increase in PKA activity in response to Hh. However, we saw no change in membrane CAMP levels with a comparable myristoylated CAMP biosensor. It may be that the EPAC-BRET biosensor is not sensitive enough to detect relatively small changes in CAMP levels in response to activation of endogenous signaling, where negative feedback may also limit the magnitude of the response. Alternatively, these cells may differ in some other way from the other cell lines previously tested (discussed below). Nevertheless, hyperactivation of the pathway at the level of Smo produced a substantial CAMP response. The fact that Gαs depletion blocked the increase in CAMP levels strongly suggests that it is due to increased CAMP production rather than a decreased rate of degradation by phosphodiesterases. The relatively slow accumulation of CAMP over several hours is somewhat unusual, as GPCRs typically activate heterotrimeric G protein signaling on a time scale of seconds to minutes. However, it fits with the previously characterized prolonged time frame for Smo activation (59), which involves phosphorylation by PKA and Cki as well as trafficking to the plasma membrane.
At the level of Smo, we identified several features required for this response. The ability of Smo to trigger Go\alpha-dependent cAMP production lies within its highly conserved core. The seven-transmembrane domain is not sufficient, as the C-terminal 26 amino acids of Smo\textsubscript{core} (between amino acids 626 and 651), situated at the end of its cytoplasmic tail, are required for the effect. This same region is required for Smo\textsubscript{core} to interact with Cos2 and to promote expression of Hh target genes (11). However, we did not observe a link between HSC-dependent signaling and the cAMP response. In fact the ability of CSBD, which interferes with the interaction between Smo and Cos2 (50), to destabilize Smo\textsubscript{SD} and block target gene expression and yet not affect the cAMP response suggests that the two responses may be mediated by separate pools of Smo protein. Interestingly, mutation of the N-linked glycosylation sites in mouse Smo impaired its ability to signal through Go\alpha without affecting canonical signaling, pointing to the possible existence of a specific Go\alpha-signaling state of Smo in mammalian cells (60). Like signaling through the HSC, the cAMP response is Hh-activated and inhibited by Ptc, suggesting that activation of
Smo by PKA and CkI is required. Crucially, however, whereas PKA/CkI phosphorylation is sufficient for activation of Hh target gene expression, the cAMP response is strictly dependent upon SmoCT phosphorylation by Gprk2. This is reminiscent of the phenomenon of phosphorylation barcoding that has been described for some GPCRs, where phosphorylation at different sets of sites in the receptor tail by different kinases dictates distinct signaling responses (61).

Given these constraints, how could Smo activity lead to signaling through Gαs? The most straightforward model would be that Smo directly couples to Gαs like a typical GPCR. If so, then we could explain the difference between our observations and others’ demonstration of Smo–Gαi coupling as a cell-type or context-dependent ability of Smo to couple to different Gα proteins, as observed for many GPCRs (62). However, we have been unable to obtain compelling evidence for a direct interaction between Smo and Gαi. We favor the alternative explanation that Smo regulates a GPCR that couples to Gαs in S2 cells. Gpr161 provides a precedent for this in mammalian cells. The ability of Gpr161 to localize to the cilium where it can couple to Gαs is determined by the activity of Smo in the cilium (63), thus providing an indirect link between Smo and Gαs. Interestingly, there is some evidence that Smo is trafficked to the cilium in response to phosphorylation by GRK2 in mammalian cells (15) (although this has been disputed) (16). In Drosophila, Gprk2 is required for internalization of Smo from the plasma membrane in response to Hh (64). Thus it could be that the regulation of Smo–Gαs signaling by Gprk2 that we observe in S2 cells reflects GRK-dependent trafficking of Smo to a subcellular location where it regulates a Gαs-coupled GPCR, perhaps on endosomes (from which mammalian GPCRs have increasingly been shown to signal through heterotrimeric G proteins after GRK-dependent internalization) (65). If this is the case, the difference in Gαs signaling between S2 cells and other insect cell lines could reflect differences in the GPCRs they express.

Regardless of whether Smo directly or indirectly regulates Gαs in S2 cells, we find that Gαs does play a role in the endogenous Hh response. Because Smo activation leads to localized PKA activity at the plasma membrane (45), we imagined that a localized burst of cAMP production might be required to enhance Smo phosphorylation to allow high-threshold target gene expression. However, our results suggest that such a mechanism is not required for high-threshold signaling. Instead, we consistently see selective impairment of low-threshold responses when Gαs is depleted or mutated in S2 cells, in wing disc clones, and most clearly, throughout an entire compartment. The narrowing of the domain of dpp expression in response to gas-depletion suggests that the sensitivity of cells to low levels of Hh was reduced, consistent with the impairment of ptc-luc reporter activation we observed in S2 cells in response to low but not high levels of Hh. Thus, rather than boosting Smo activity toward its maximum, Gαs-driven cAMP production may be most important for enhancing Smo activation above a signaling threshold under limiting ligand conditions. Interestingly, while this manuscript was under review, Pusapati et al. (66) reported similar findings in vertebrates. They showed that GPCR signaling through Gαs, downstream of both Smo and GRKs, regulates the sensitivity of responding cells to Sonic Hedgehog. The parallel between these findings and ours indicates that regulation of cAMP in target cells is an evolutionarily conserved mechanism for fine-tuning Hh responsiveness.

Finally, in contrast to previously published work, we observe an inhibitory rather than stimulatory role for Gαi in the Hh response. Specifically, Gαi depletion led to ectopic expression of Hh target genes in cells located away from the A/P boundary, where ligand levels are lower. This difference from previous work may be due to a difference in the extent of signaling impairment in Gαi-depleted cells versus gai mutant clones, similar to the way that PKA inhibition can suppress the Hh response (8), whereas complete elimination of the catalytic subunit leads to constitutive target gene expression (31). In any case, these results are consistent with our Gαi depletion experiments and other work showing a positive role for Gαi/cAMP in the Hh response (36, 67, 68). Together they clearly suggest a positive role for regulated cAMP production in controlling the sensitivity of cells to Hh.

**Experimental procedures**

**Expression constructs**

Expression plasmids encoding C-terminally–tagged GFP Smo mutants and Smo truncations under control of the metallothionein promoter (in pRmHa3.puro) were previously described (11). The expression construct for Smo^ΔC (lacking the C-terminal-most 52 amino acids containing a Fu-binding domain) (49) was generated by PCR amplification of a portion of the Smo coding sequence spanning the EcoRI site at codon 797–798 to the Leu-984 codon. Primers were designed to introduce a 3’ stop codon and NotI site immediately following codon 984. The resulting EcoRI-NotI fragment was used to replace the corresponding C-terminus–encoding fragment in a pRmHa3.puro backbone containing the Smo^SD coding sequence fused to C-terminal GFP (engineered as a cassette flanked by NotI and KpnI restriction sites). To create the Cos2 Smo-binding domain (CSBD) expression construct (40), sequences encoding amino acids 1001–1201 of Cos2 were PCR amplified, introducing a 5’ EcoRI and 3’ KpnI site. The resulting EcoRI-KpnI restriction fragment was cloned into a modified pRmHa3.puro plasmid encoding an in-frame N-terminal Myc epitope tag. For expressing Ptc, an EcoRI-KpnI fragment encoding full-length Ptc fused to a C-terminal GFP tag (obtained from Stephen Cohen) was cloned into pRmHa3.puro. The plasmids encoding the EPAC-BRET biosensor or the same protein lacking the GFP10 moiety (used as a control for background emission), as well as plasmids encoding constitutively active GαsQ215L and GαiQ202I, were prepared as described (36). pRmHa3.puro/HhN, which encodes an active N-terminal fragment of Drosophila Hh plasmid (59), was used in the preparation of HhN-conditioned media and for co-transfection of cells transfected with Smo expression plasmids in some experiments. An empty pRmHa3.puro vector (−) was used for transfection as a control.

**BRET and ptc-luciferase reporter assays, dsRNA treatment**

EPAC-BRET assays were performed essentially as described (36). Briefly, for BRET experiments not involving dsRNA treat-
GRK-phosphorylated Drosophila Smo activates Gαs signaling

For time course experiments, Smo-expressing S2-R+ cells were treated with medium conditioned by either control cells or cells expressing HhN as previously described (64). Cells were transfected with 150 ng each of pMT.puro/GFP10-EPAC-RLucII/T781A,F782A, pMT.puro/SmoWT-GFP, and (in the control cells) pMT.puro/HhN and processed as above. On day 4, growth medium was gently removed and 0.1 ml of either control or HhN-conditioned media was added to each well at the appropriate time. Cells treated at different intervals were processed simultaneously by adding DeepBlueC directly to the conditioned media and BRET values were determined as above. Time-dependent changes in SmoWT phosphorylation following exposure to control- or HhN-conditioned media were determined by immunoblotting with rabbit α-GFP antibody (Torres Pines Scientific) as described (11). In other experiments, immunoblotting was performed with mouse monoclonal anti-myc (9E10; Santa Cruz Biotechnology), mouse monoclonal anti-α-tubulin (12G10; obtained from the Developmental Studies Hybridoma Bank (DSHB)), guinea pig anti-Gprk2 (64), or guinea pig anti-Smo.

Drosophila crosses, immunostainings, and image analysis

For generating gas mutant clones, we used the gas^{R60A} mutant allele (stock obtained from the Bloomington Drosophila Stock Centre). This allele contains a mutation that converts Tyr-231 to a stop codon, deleting the C-terminal one-third of the protein, and should be a null allele. To make gas clones in a Minute background, winf^{10638},FRT42D,gas^{R60A}/CyO,Kr-GAL4, UAS-GFP males were crossed at 25 °C to w,hsFLP; FRT42D, Ub::GFP,M(2R)S3'/CyO virgins. For WT clones, winf^{10638}, FRT42D males were used. Offspring from the two crosses were heat shocked at 37.5 °C and dissected in parallel. For no clones control, the heat shock was omitted. Wing disc-bearing anterior halves were dissected from wandering third instar larvae and processed for immunofluorescence staining with rabbit anti-β-gal antibody (Santa Cruz Biotechnology). To quantify clonal growth, the total area of the wing pouch and of the GFP-negative regions contained in the wing pouch for 10 discs were measured using the Histogram function of Photoshop. Clone size was expressed as the total GFP-negative clonal area over total wing pouch area.

For Gαs and Gαi depletion experiments w;UAS-Dcrp-GAL4,dpp^{10638}/CyO or w;UAS-Dcrp-nub-GAL4,dpp^{10638}/CyO females were crossed at 27 °C to w,UAS-GFP males (control), w;UAS-Gas.dsRNA males (from Vienna Drosophila Resource Centre; stock number v24958), or w;UAS-Gai.dsRNA males (69) (kindly provided by J. Knoblich). Wandering larvae were dissected and anterior and anterior halves processed for immunofluorescence with rabbit anti-β-gal, rat anti-Ci (2A1 from DSHB), and mouse anti-βtubulin (12G10; obtained from a monoclonal anticytokeratin mouse antibody (64)). Discs were viewed using confocal microscopy as described (11). To quantify fluorescence, boxes of equal size spanning the A/P boundary were drawn in the dorsal and ventral regions of each wing disc image and fluorescence values along the anterior-posterior axis were calculated using the Plot Profile function of ImageJ. Dorsal and ventral data were normalized by dividing each data point by the average maximum intensity value from the ventral compartment of that disc (defined as the average of the 10 highest values), effectively converting them to % maximum WT response. Data were then arranged to align the A/P boundaries and pixel-by-pixel data from four discs was averaged to yield a mean intensity plot.

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