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A G protein functions immediately downstream of Smoothened in Hedgehog signaling

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Abstract

The Hedgehog (Hh) signaling pathway plays an evolutionarily conserved role in patterning fields of cells during metazoan development, and is inappropriately activated in cancer1,2. Hh pathway activity is absolutely dependent upon signaling by the seven-transmembrane protein Smoothened (Smo), which is regulated by the Hh receptor Patched (Ptc). Smo signals to an intracellular multi-protein complex containing the Kinesin related protein Costal2 (Cos2), the protein kinase Fused (Fu) and the transcription factor Cubitus interruptus (Ci)3. In the absence of Hh, this complex regulates the cleavage of full length Ci to a truncated repressor protein, Ci_{75} in a process that is dependent upon the proteosome and priming phosphorylations by Protein Kinase A (PKA)4. Binding of Hh to Ptc blocks Ptc-mediated Smo inhibition, allowing Smo to signal to the intracellular components to attenuate Ci cleavage. Because of its homology with the Frizzled family of G protein coupled receptors (GPCR)5, a likely candidate for an immediate Smo effector would be a heterotrimeric G protein. However, the role G proteins may play in Hh signal transduction is unclear and quite controversial6-10, which has led to widespread speculation that Smo signals through a variety of novel G protein independent mechanisms. Here, we present *in vitro* and *in vivo* evidence that Smo activates a G protein to modulate intracellular cyclic AMP (cAMP) levels in response to Hh. Our results demonstrate that Smo functions as a canonical GPCR, which signals through Gαi to regulate Hh pathway activation.

In order to examine whether a G protein is involved in Hh signaling, we targeted a series of G proteins by double stranded RNA (dsRNA)-mediated knockdown. Clone-8 (Cl8) cells

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Author contributions. S.K.O was involved in the design, execution and analysis of experiments, and in writing the manuscript. D.L.F assisted in design and execution of dsRNA experiments and sequencing of *Gai* alleles. J.H. was involved in design and execution of cAMP assays. N.S.S. assisted in execution of cAMP assays. Y.F.A. consulted on design and interpretation of genetic experiments. D.J.R. was involved in design and analysis of experiments and writing the manuscript.

were treated with control or Gα subunit specific dsRNA and assayed for changes in Hh-mediated induction of a *ptc-luciferase* reporter construct 11. We find that while Gαs and Gαo dsRNAs do not significantly alter Hh-induced reporter activation (Sup. Fig. 1a-b), Gαi knockdown is able to trigger a decrease in Hh dependent reporter gene expression (Fig. 1a). While not as effective as Smo knockdown in silencing Hh reporter gene activation (Sup. Fig. 1c), Gαi dsRNA specific to the coding sequence, or 3′ UTR, reduces Hh-induced reporter activity by approximately 70% (Fig. 1a), supporting a role for Gαi in the Hh pathway. To confirm the specificity of Gαi dsRNA effects, we established the functional IC_{50} of Gαi 3′ UTR dsRNA (Sup. Fig. 1e), then attempted to rescue reporter activity through ectopic expression of wild type *Gαi* or constitutively active *Gαi*^{Q205L}. Hh-stimulated reporter activity can be restored by both wild type and constitutively active Gαi (Fig. 1b), confirming the specificity of our Gαi dsRNA-mediated effects. Western blot analyses of Cl8 lysates reveal that cells treated with Gαi dsRNA show attenuated stabilization of Ci (Fig. 1c, compare lanes 2 and 4) and decreased Fu phosphorylation (Sup. Fig. 1d, compare lanes 2 and 4) in response to Hh. Hh-induced Smo phosphorylation is maintained in the presence of Gαi dsRNA (Fig. 1c, arrow), supporting that Gαi functions downstream of Smo and upstream of Fu and Ci.

To determine whether Gαi can modulate Hh pathway activity *in vivo*, *Gαi* constructs were expressed in wing imaginal discs using *MS1096-Gal4* or *C765-Gal4*. Expression of an inactive *Gαi* mutant (*Gαi*^{G204A}) or wild type *Gαi* has little effect on wing vein patterning (Fig 2a-c). However, expression of constitutively active *Gαi*^{Q205L} 12 results in widening of longitudinal vein LV3-LV4 spacing and ectopic vein material on LV2 and LV3 (Fig. 2d compared to 2a-c). The severity of this phenotype is dose-dependent, as higher level expression of *UAS-Gαi*^{Q205L} triggers more severe ectopic vein material anterior to LV3, and further widening of LV3-LV4 spacing (Fig. 2e). Expression of *Gαi*^{Q205L} in wing imaginal discs also results in over-growth of the wing pouch, along with expansion of full length Ci (Fig. 2g, arrow, compared to 2f, no driver). This Ci expansion triggers ectopic expression of the Hh target gene *decapentaplegic* (*dpp)*13,14 in the wing pouch, as evidenced by a *dpp-lacZ* reporter gene (Fig. 2g′ compared to 2f′). Gαi-mediated ectopic expression of *dpp* is consistent with the ectopic veins observed in wings expressing *Gαi*^{Q205L} (Fig. 2e, arrows)15. Taken together, these results support a role for activation of Gαi in regulating the stability of Ci, and link Gαi to regulation of a known Hh target gene.

To determine whether Gαi functions downstream of Smo *in vivo*, we analyzed the ability of Gαi to modulate Hh pathway activity in a *smo* sensitized background. As previously demonstrated, expression of a dominant negative *smo* transgene, *UAS-Smo5A*, results in severe disruption of LV3-LV4 wing patterning (Fig. 2h and 16). Expression of wild type Gαi in this *smo* sensitized background allows for partial rescue of wing vein structures in the LV3/LV4 zone (Fig. 2i). Expression of constitutively active *Gαi*^{Q205L} results in a more complete rescue of the Hh loss of function phenotype, allowing for near total restoration of LV3/LV4 patterning (Fig. 2j). As a control, *UAS-GFP* was co-expressed with *Smo5A*, and found to have no effect on the Smo5A-induced phenotype (data not shown).

To examine the ability of Gαi^{Q205L} to modulate Ci stability and Hh target gene activation in the *smo* sensitized background, wing imaginal discs were immunostained with antibodies that recognize full length Ci and the target gene product Ptc. *UAS-Smo5A* expression results in decreased *ptc* expression and disruption of the Ci gradient (Sup. Fig. 2b compared to 2a). Expression of constitutively active $G\alpha i^{Q205L}$ in this *smo* sensitized background results in partial restoration of the Ci gradient and a near-complete rescue of *ptc* expression at the anterior/posterior (A/P) border (Sup Fig. 2c). These results support the model that Gαi contributes to the regulation of Hh target gene expression and Ci stability. Further, that this regulation occurs when Smo function is compromised suggests that Gαi affects Hh signaling at a level downstream of Smo.

To determine whether Gαi is required for Hh signaling *in vivo*, we examined Hh target gene expression in clones of cells homozygous for $G\alpha i$ mutation. The null allele $G\alpha i^{P20}$ removes the entire coding region of the $G\alpha i$ gene, and is homozygous lethal17. $G\alpha i^{P8}$ is a putative hypomorph, which removes the bulk of exons 1 and 2, but leaves the transcriptional start site intact and produces a transcript (Sup. Fig. 3b). Flies that are homozygous for the $G\alpha i^{P8}$ mutation are viable, but unhealthy17. Mosaic analysis reveals that expression of the Hh target gene *dpp* is decreased in both $G\alpha i^{P20}$ (Fig. 3a) and $G\alpha i^{P8}$ (Fig. 3b) mutant clones, supporting a role for Gαi in activation of Hh target genes *in vivo*. To confirm that effects on *dpp* expression are due to loss of $G\alpha i$, we attempted to rescue $G\alpha i^{P20}$ null clones with *UAS-*$G\alpha i$. We find that ectopic expression of $G\alpha i$ is able to rescue *dpp* reporter gene expression in $G\alpha i^{P20}$ clones (Fig. 3c-c*), consistent with decreased *dpp* expression resulting from disruption of $G\alpha i$.

To determine whether compromised Gαi activity alters Hh-dependent patterning, we utilized the viable mutant allele $G\alpha i^{P8}$, and an additional viable allele described to be a null or strong hypomorph18, $G\alpha i^{57}$. We find that whereas homozygous $G\alpha i^{P8}$ and $G\alpha i^{57}$ mutants do not exhibit vein fusions that are typical of strong Hh loss of function, their wings are smaller than wild type wings (Fig 3d and Sup. Fig. 3c). Small wing size might result from altered *dpp* expression in anterior cells of the wing pouch, as Dpp regulates wing blade size19,20. Additionally, we find that both $G\alpha i^{P8}$ and $G\alpha i^{57}$ mutant flies demonstrate varying degrees of incomplete thorax closure, as evidenced by mild to severe thoracic clefts (Fig. 3e′, arrow, compared to 3e and Sup. Fig. 3a-a*,arrows). This phenotype is also consistent with decreased *dpp* expression, in that Dpp, in conjunction with JNK signaling, controls spreading of the anterior edge of wing imaginal discs to initiate thorax closure21,22. To confirm that this phenotype results from decreased Hh signaling, we expressed *ptc* in the notum and dorsal compartment of the wing imaginal disc. *ptc* expression triggers the formation of a thoracic cleft when expressed under control of *pannier* and *apterous* promoters (Fig. 3e*, arrow, compared to 3e′ and 3e and data not shown), suggesting that the thoracic phenotype we observe in $G\alpha i$ flies results from compromised Hh signaling. Because $G\alpha i^{P20}$ null mutant animals are not viable, we could not examine their wings or thoraces. However, we find that attenuation of Hh signaling by expressing dominant negative *Smo5A* is enhanced in $G\alpha i^{P20}$ heterozygotes, as evidenced by disruption of LV3 (Sup. Fig. 3d compared to d′).

Our *in vitro* and *in vivo* data suggest that loss of Gαi might compromise Ci stabilization in Hh-receiving cells. Interestingly, when we examine Ci and Smo levels in $G\alpha i$ mutant clones, we find that both appear to be increased in a cell autonomous manner (Sup. Fig. 3e and f).

These results are consistent with the modest stabilization of Smo and Ci that we observe upon *in vitro* Gαi knock-down in non-Hh treated cells (Fig. 1c, compare lane 1 with 3). While these results are unexpected, since Gαi loss is predicted to increase PKA activity and Ci degradation, previous studies have demonstrated that PKA functions to both positively and negatively regulate Hh signaling 23. Phosphorylation of Smo by PKA plays a positive role in pathway activation24, and might account for the modest stabilization of Ci we observe.

If Smo signals through Gαi, it should be able to induce Gαi activation rapidly in response to Hh stimulation. To assay for Hh-mediated activation of Gαi, we treated Cl8 cells with conditioned media containing the amino-terminal Hh signaling molecule (HhN) or control conditioned media, then assayed for Hh-induced changes in intracellular cAMP. We find that within 5-10 minutes, HhN treatment reduces the basal intracellular cAMP concentration by approximately 50% (Fig. 4a). To confirm that the Hh-induced decrease in intracellular cAMP is dependent upon Hh signaling through Smo and Gαi, we treated cells with Smo, Gαi or control dsRNA, then assayed for a Hh-induced decrease in cAMP (Fig. 4b). We find that whereas cells transfected with control dsRNA maintain the ability to decrease intracellular cAMP in response to HhN, cells transfected with either Smo or Gαi dsRNA are attenuated in their ability to do so. Taken together, these results support that Gαi is activated rapidly, in a Smo-dependent manner, in order to modulate cAMP levels in response to Hh.

To determine whether modulation of cAMP can alter Hh signaling *in vivo*, we utilized a hypomorphic mutant allele of the cAMP-specific phosphodiesterase *dunce* (dnc^1)25 to raise intracellular cAMP levels in a Hh independent manner. Hemizygous dnc^1 animals are viable with no obvious Hh defects (Fig. 4c). However, introduction of the dnc^1 mutation into a *smo* sensitized background enhances the Smo loss of function phenotype to result in wings with near complete elimination of wing vein patterning (Fig. 4e compared to d). This enhanced Hh loss of function phenotype is similar to the phenotype obtained upon decreasing *smo* gene dosage by one-half in the same *smo* sensitized background (compare Fig. 4f with e and d). Along with our *in vitro* cAMP assays, these results support that Hh activates Smo to modulate intracellular cAMP, via Gαi, and that this function is important for proper pathway activity *in vivo*.

Cos2 associates with membranes, microtubules, PKA, Smo, Fu and Ci26,27. To determine whether Cos2 facilitates the coupling of Gαi with these Hh signaling components, we prepared lysates from cells expressing *HA-Gαi*, and then immunoprecipitated Cos2 (Fig. 4g). We find that Gαi associates with the Cos2 complex, and that this association is enriched in response to Hh (Fig. 4g, left panel, compare lane 7 to 5). The binding of Fu to Cos2 is not altered by Hh, suggesting that the recruitment of Gαi to this protein complex is regulated. This result suggests that Cos2 facilitates the coupling of Smo with Gαi and additional downstream effectors necessary to transduce the Hh signal.

Here, we have shown a requirement for Gαi in the Hh signaling pathway. We have demonstrated Hh-mediated recruitment and activation of Gαi that results in decreased intracellular cAMP, suggesting that Hh may regulate PKA through modulation of intracellular cAMP concentration. We have also demonstrated that Gαi can modulate Hh pathway activity *in vitro* and *in vivo*, and appears to do so at a level downstream of Smo. Furthermore, loss of Gαi alters Hh signaling *in vivo*, supporting that Gαi is a requisite member of the Hh pathway.

Methods Summary

All cell-based assays described here were performed in Clone-8 (Cl8) cells, which are a Hh responsive wing imaginal disc cell line. T7 DNA templates were generated from Gα cDNAs by PCR using Vent polymerase (NEB) as described 28. Cells were lysed 72 hours post dsRNA transfection 29 and post-nuclear lysates analyzed by immunoblotting, using the indicated antibodies. Reporter assays were performed in cells transfected with the indicated agents, along with a *ptc-luciferase* reporter construct 11 and *act-renilla* transfection control 29. cAMP levels were determined from cells treated with or without HhN conditioned media for 5-10 minutes, using a [^{3}H]-cAMP receptor competition assay 30. Measured cAMP levels were then compared to a standard curve to determine intracellular cAMP. For Gαi and Smo knockdown effects on cAMP production, cells were transfected with control, Smo or Gαi specific dsRNA 72 hours prior to treatment with HhN or control conditioned media. Student's T test was used to determine p values. Error bars indicate standard error of the mean.

Fly stocks were maintained on standard yeast-cornmeal molasses media. For dnc^1 enhancement of the Smo5A phenotype, crosses were begun at 25° C, then moved to 22° C for pupation. All other crosses were performed at 25° C with the exception of mosaic analyses, which were performed at room temperature. Mutant clones were marked by loss of GFP expression. Imaginal discs were dissected and immunostained as described previously29. Adult wings were mounted on glass microscope slides using DPX mounting media. Wing images were collected using a Zeiss Axioskop2 microscope. All images were processed using Adobe Photoshop 6.0. The $G\alpha i^{P8}$ breakpoint, as well as expression of the other *Gαi* alleles, was determined using standard molecular biology techniques.

Methods

RNA interference

T7 DNA templates were generated from Gα cDNAs by PCR using Vent polymerase (NEB) as described 28. dsRNA was generated from these T7 templates using the Megascript T7 kit (Ambion) per manufacturer's instructions. Gα subunit specific dsRNAs were generated from the following regions of the indicated G protein cDNAs: Gαi, basepairs 219-966 or 14-538 of the 3′ UTR, Gαo, basepairs 213-964 and Gαs, basepairs 208-1059. BLAST queries were performed to confirm that designed dsRNAs were subunit specific for the intended α subunit. Gαi cDNA was obtained from the Drosophila Genomics Resource Center (DGRC), Gαo cDNAs were provided by the DGRC and T. Kornberg (UCSF), and Gαs cDNA was provided by E. Lee (Vanderbilt). Semi-quantitative rtPCR was performed to confirm Gαo and Gαs knockdown. RNA was purified using TriReagent (MRC) per manufacturer's instructions. cDNA was generated from 5 μg total RNA using random primers and Superscript II reverse transcriptase (Invitrogen). One-twentieth of the cDNA was used for

PCR of G proteins and Actin loading control. Reverse transcriptase minus reactions were used as a control.

To examine the effects of Gαi and/or Smo knockdown on the Hh signaling pathway, Cl8 cells were lysed 72 hours post dsRNA transfection as previously described 29. Post-nuclear lysates were analyzed by SDS-PAGE and western blot using the following published antibodies: Ci 31, Cos2 32, Fu33, Gαi12 and Kinesin as a loading control (Cytoskeleton, Inc.). To generate the Smo antibody used for western blot analysis, rats were injected with a peptide corresponding to amino acids 1010-1030 of the Smo carboxyl-terminal tail. Antibodies were then affinity purified from sera over a peptide column. Peptides and antibodies were produced and purified using the Covance custom antibody service.

Reporter assays

Clone-8 (Cl8) cells were transfected with Gαi dsRNA, *ptc-luciferase* reporter construct 11 and *act-renilla* transfection control 29 using Cellfectin transfection reagent (Invitrogen). Relative luciferase activity was determined 72 hours post transfection using the Dual Luciferase Assay System (Promega). Each assay was performed a minimum of three times, in duplicate. Values were averaged to determine *ptc-luciferase* activity relative to *renilla* transfection control. Error bars indicate standard error of the mean.

Fly crosses and transgenes

Fly stocks were maintained on standard yeast-cornmeal molasses media. For dnc^1 enhancement of the Smo5A phenotype, crosses were begun at 25° C, then moved to 22° C for pupation. All other crosses were performed at 25° C with the exception of mosaic analyses, which were performed at room temperature. The following genotypes were used for generation of $G\alpha i^{P20}$ and $G\alpha i^{P8}$ mitotic clones: *w hs-flp;*$G\alpha i^{P20}$ *FRT2A/Ubi-GFP FRT2A* and *w hs-flp;*$G\alpha i^{P8}$ *FRT2A/Ubi-GFP FRT2A*. The following genotype was used for $G\alpha i^{P20}$ clone rescue experiments: *MS1096-Gal4/yw hs-flp; dpp-lacZ/UAS-Gαi;* $G\alpha i^{P20}$ *FRT2A/Ubi-GFP FRT 2A*. Recombination was induced by incubation at 37°C for 1 hour, 48 hours post egg laying34. Null mutant clones were marked by loss of GFP expression. Generation of *UAS-*$G\alpha i^{G204A}$ transgenic flies was performed by the Duke Model Systems Genomics Core Injection service. *UAS-Gαi* and *UAS-*$G\alpha i^{Q205L}$ flies were provided by J. Hooper (UCHSC). *C765-Gal4, UAS-Smo5A*, flies were provided by D. Casso and T. Kornberg (UCSF). *smo* alleles were provided by D. Kalderon, K. Basler and P. Ingham. *Gαi* alleles were provided by X. Yang, W. Chia and J. Knoblich. dnc^1*, dpp-lacZ, hsFLP; Ubi-GFP*, *FRT2A*, *pannier-Gal4* and *MS1096-Gal4* flies were obtained from the Bloomington Drosophila Stock Center.

PCR and sequencing

To sequence the $G\alpha i^{P8}$ breakpoint, one adult fly was homogenized in 50 μl SB buffer (10mM Tris (pH 8.2), 25mM NaCl, 1mM EDTA, 0.2mg/ml protease K) and incubated for 30 minutes at 37°C, followed by an additional 10 minutes at 85°C. Lysates were centrifuged for 5 minutes at 2000 × g. Four μl of the supernatant was used as template for PCR with forward primer (GGATCATATGAGTGGCATTCAAGC) located -245 to -222, relative to the *Gαi* transcription start site, and reverse primer (CTGATAGCGCGACGCAGAAG), basepairs + 6197 to + 6216 relative to the transcription start site. Ten ng of PCR product was used for sequencing. The *Gαi*^{P8} mutation deletes basepairs +29 to +6104 with respect to the *Gαi* transcription start site. For semi-quantitative RT-PCR analysis, 3 adult wild type or *Gαi*^{P8} flies were homogenized in 200μl Tri Reagent and RNA was extracted per manufacturer's instructions (Molecular Research Center). Approximately 2 μg of RNA was used for reverse transcription reactions, using random hexamer oligos and SuperScript II reverse transcriptase (Invitrogen), or RT-minus control reactions.

Immunostaining and microscopy

Imaginal discs were dissected and immunostained as described previously29. Discs were stained using Ci (2A1)31, Ptc15, En35, Gαi 12, Smo36 and β-galactosidase (Promega) primary antibodies and Alexa-fluor conjugated secondary antibodies (Molecular Probes), as described29. Adult wings were mounted on glass microscope slides using DPX mounting media. Wing images were collected using a Zeiss Axioskop2 microscope and processed with Adobe Photoshop 6.0.

Immunoprecipitation

Cl8 cells were transfected with *pAct-HA-Gαi*, *pAct-hh* or empty vector control. Cells were lysed 48 hours post transfection and lysates were immunoprecipitated using Cos2 antisera as previously described32,37. HA antibody was obtained from GeneTex, Inc.

cAMP assay

Cl8 cells were treated with HhN or control S2 conditioned media for 10 minutes prior to lysis by boiling in 50 mM Tris-HCl, 4 mM EDTA, pH 7.9. Cell lysates were cleared of nuclei and cellular debris by centrifugation at 2000 × g for 10 minutes at 4° C. cAMP levels in cell lysates were determined by a radio-receptor competition assay as previously described30. Lysates were compared against a known standard curve similar to Sup. Fig. 4 to determine intracellular cAMP. For Hh-mediated effects on intracellular cAMP, the assay was performed two times in duplicate, and all data were averaged. For Gαi and Smo knockdown effects on cAMP production, cells were transfected with control, Smo or Gαi specific dsRNA 72 hours prior to treatment with HhN or control conditioned media. Following treatment with conditioned media for 5-10 minutes, cells were lysed and analyzed as described above. Assays were performed 2 times in triplicate for Gαi and 3 times in triplicate for Smo. A representative assay is shown. Student's T test was used to determine p values. Error bars indicate standard error of the mean.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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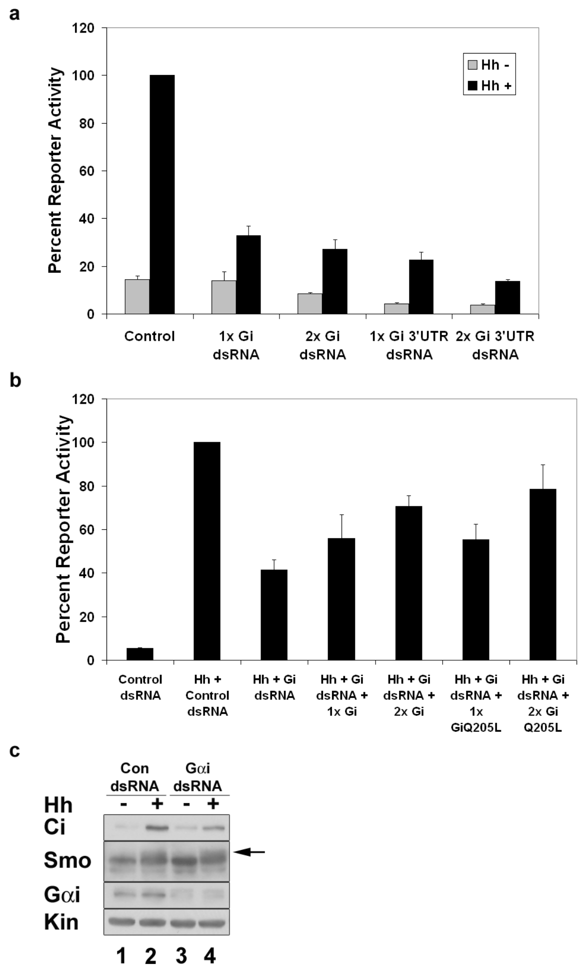

Figure 1.

Gαi is required for Hh signaling. **a-b.** Cl8 cells were transfected with *ptc-luciferase*, *act-renilla*, *hh* expression vector or empty vector control, and the indicated dsRNA and/or *Gαi* expression vectors. Percent reporter expression relative to maximal Hh activity for control dsRNA is shown. **c**. Cl8 cells were transfected with control or *Gαi* dsRNA and *hh* expression vector or empty vector control. Cell lysates were analyzed by immunoblotting with the indicated antibodies. The arrow marks the phosphorylation-induced mobility shift. Kinesin (Kin) serves as a loading control. Error bars indicate s.e.m.

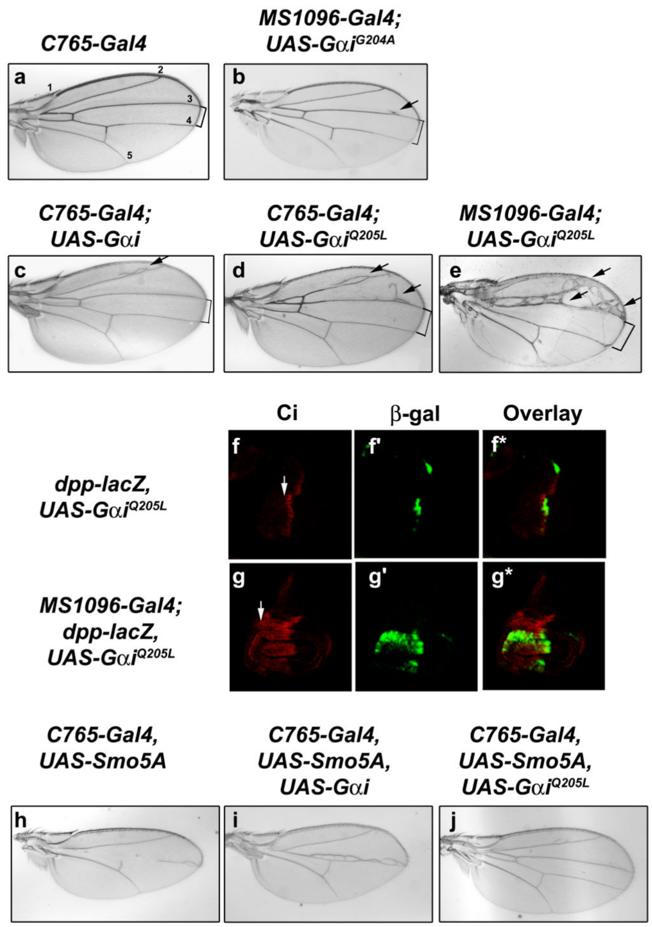

Figure 2.

Gαi expression results in ectopic Hh signaling. **a-g**. Longitudinal veins are numbered (**a**). Wings from (**b**) *UAS-Gαi*^{G204A}, (**c**) *UAS-Gαi* (**d, e**) *UAS-Gαi*^{Q205L} demonstrate ectopic veins (arrows) and LV3-4 widening (brackets), as compared to control (**a**). Wing discs from (**f**) *dpp-lacZ, UAS-Gαi*^{Q205L} and (**g**) *dpp-lacZ, UAS-Gαi*^{Q205L}, *MS1096-Gal4* larvae were immunostained for Ci (red) and β-galactosidase (green). Overlays are shown in **f*** and **g***. Anterior is left and dorsal is up. **h-j.** Expression of wild type *Gαi* partially rescues, and *Gαi*^{Q205L} fully rescues, the phenotype of induced by dominant negative *UAS-Smo5A* (compare **i** and **j** to **h**).

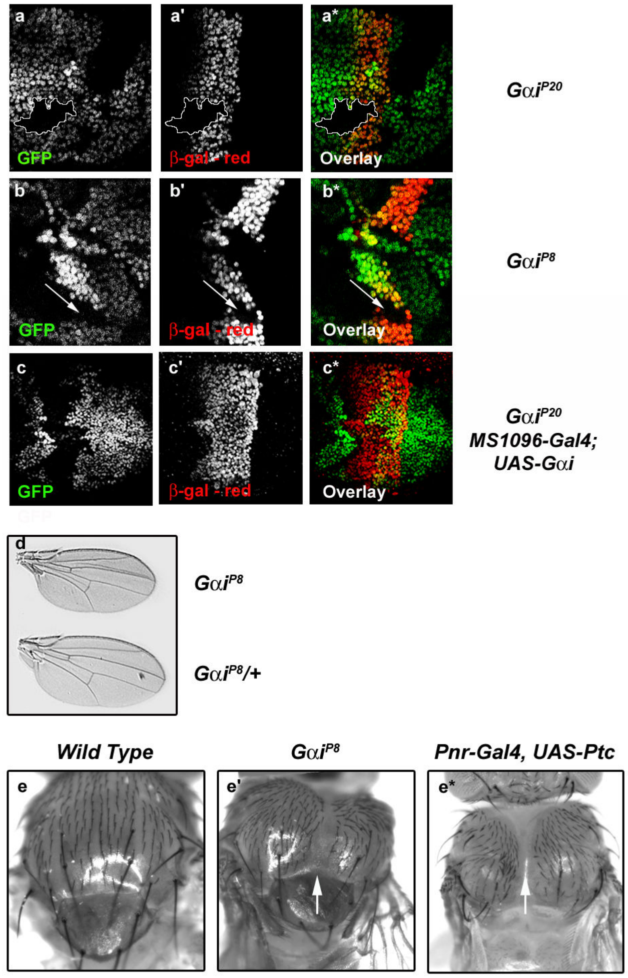

Figure 3.

Gαi is required for Hh signaling *in vivo*. **a-c**. Mitotic clones were generated with the $G\alpha i^{P20}$ null (**a, c**) or $G\alpha i^{P8}$ hypomorphic (**b**) alleles. Wing discs were stained for the *dpp-LacZ* gene product β-gal (red). Loss of *GFP* expression (green) marks $G\alpha i$ mutant clones. **c**. $G\alpha i^{P20}$ null clones were generated in *MS1096-Gal4*, *UAS-*$G\alpha i$ wing discs. **d-e.** $G\alpha i$ phenotypes are consistent with decreased Hh signaling. **d.** Homozygous $G\alpha i^{P8}$ mutant flies exhibit a small wing phenotype. **e′**. $G\alpha i^{P8}$ and **e*** *pnr-Gal4*, *UAS-ptc* flies have thoracic clefts (arrows), as compared to (**e**) wild type.

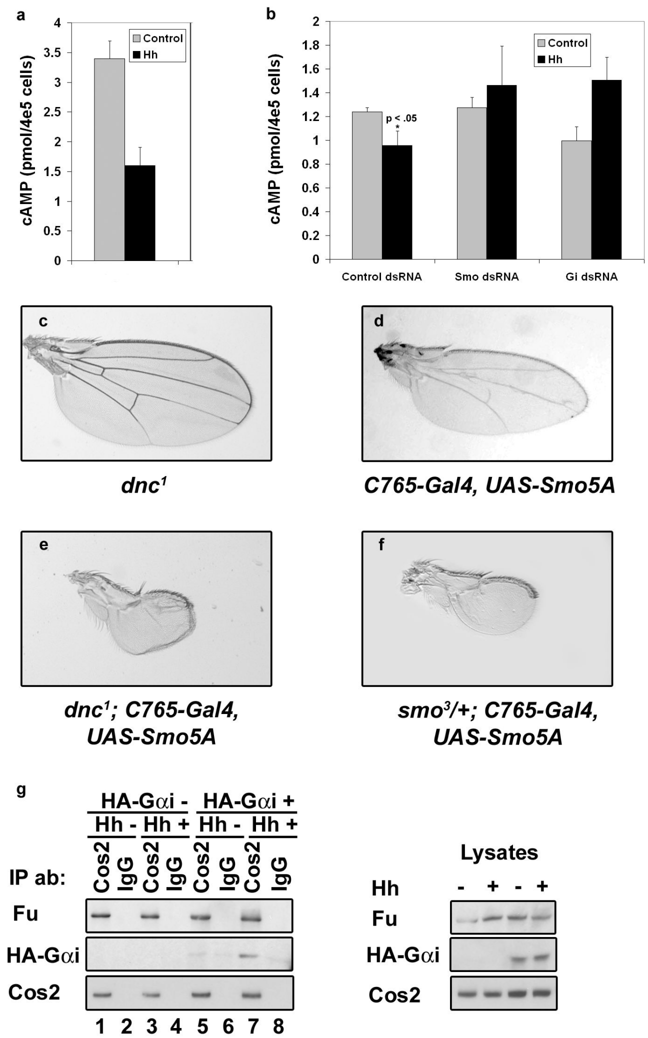

Figure 4.

Hh regulates Gαi activity and association with Cos2. **a**. Cl8 cells were treated with control or HhN conditioned media. Error bars indicate s.e.m. **b**. Cl8 cells were transfected with control, Smo or Gαi dsRNA, and treated with HhN or control conditioned media. Wings from (**c**) hemizygous *dnc*^{1} (**d**) *UAS-Smo5A*, (**e**) *dnc*^{1}, *UAS-Smo5A* and (**f**) *smo*^{3}, *UAS-Smo5A* flies are shown. Introduction of *dnc*^{1} enhances the *Smo5A* phenotype in approximately 50% of flies (n=57). **g.** Cl8 cells were transfected with *HA-Gαi* and *hh* expression vectors, as indicated. Immunoprecipitations were performed from cell lysates using anti-Cos2 or IgG control antibodies.