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Drosophila G-protein-coupled receptor kinase 2 regulates cAMP-dependent Hedgehog signaling

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SUMMARY

G-protein-coupled receptor kinases (GRKs) play a conserved role in Hedgehog (Hh) signaling. In several systems, GRKs are required for efficient Hh target gene expression. Their principal target appears to be Smoothened (Smo), the intracellular signal-generating component of the pathway and a member of the G-protein-coupled receptor (GPCR) protein family. In *Drosophila*, a GRK called Gprk2 is needed for internalization and downregulation of activated Smo, consistent with the typical role of these kinases in negatively regulating GPCRs. However, Hh target gene activation is strongly impaired in *gprk2* mutant flies, indicating that Gprk2 must also positively regulate Hh signaling at some level. To investigate its function in signaling, we analyzed several different readouts of Hh pathway activity in animals or cells lacking Gprk2. Surprisingly, although target gene expression was impaired, Smo-dependent activation of downstream components of the signaling pathway was increased in the absence of Gprk2. This suggests that Gprk2 does indeed play a role in terminating Smo signaling. However, loss of Gprk2 resulted in a decrease in cellular cAMP concentrations to a level that was limiting for Hh target gene activation. Normal expression of target genes was restored in *gprk2* mutants by stimulating cAMP production or activating the cAMP-dependent Protein kinase A (Pka). Our results suggest that direct regulation of Smo by Gprk2 is not absolutely required for Hh target gene expression. Gprk2 is important for normal cAMP regulation, and thus has an indirect effect on the activity of Pka-regulated components of the Hh pathway, including Smo itself.

KEY WORDS: Hedgehog, Smoothened, G-protein-coupled receptor kinase, cAMP, *Drosophila*

INTRODUCTION

Hedgehog (Hh) signaling plays a conserved and crucial role in tissue growth, patterning, and maintenance. Intracellular Hh signaling is initiated by Smoothened (Smo), a G-protein-coupled receptor (GPCR) family member. In *Drosophila*, binding of Hh to its receptor complex blocks Patched (Ptc)-mediated inhibition of Smo, leading to phosphorylation of Smo by Protein kinase A (Pka) and Casein kinase I (CkI; CkIa – FlyBase), and its accumulation at the cell surface (reviewed by Jiang and Hui, 2008). Once activated, Smo signals through a canonical pathway that includes the serine/threonine kinase Fused (Fu), the kinesin-like protein Costal-2 (Cos2; Costa – FlyBase), and Suppressor of Fused (SuFu). The activity of this pathway controls the phosphorylation of the transcription factor Cubitus interruptus (Ci) by Pka and other kinases in a complex scaffolded by Cos2 and Fu, and its subsequent proteolytic processing from a full-length 155 kDa transcriptional activator form (Ci^{155}) to a 75 kDa repressor form (Ci^{Rep}) (Aza-Blanc et al., 1997; Ruel et al., 2003; Zhang et al., 2005). It also regulates nuclear entry of Ci^{155} (Methot and Basler, 2000; Wang et al., 2000). Smo-dependent activation of canonical signaling through Fu has dual effects: phosphorylation and inhibition of Cos2, leading to Ci^{155} stabilization (Lum et al., 2003; Nybakken et al., 2002); and phosphorylation and inhibition of SuFu, leading to Ci^{155} nuclear entry and transcriptional activation of target genes (Lum et al., 2003; Methot and Basler, 2000; Wang et al., 2000).

Smo may also function as a bona fide GPCR. Perhaps the strongest evidence comes from flies, where the inhibitory Gα protein, $G\alpha_i$, downregulates cAMP levels downstream of Smo in response to Hh (Ogden et al., 2008). Pka, which is one of the principal cellular targets of cAMP, plays both positive and negative regulatory roles in Hh signaling, and its constitutive activity can either increase or decrease target gene expression in different tissues (Jia et al., 2004; Li et al., 1995; Ohlmeyer and Kalderon, 1997; Tiecke et al., 2007; Zhou et al., 2006). However, it has been suggested that regulation of cAMP levels in response to Hh may not be essential for target gene activation (Jiang and Struhl, 1995; Li et al., 1995), and the general importance of G-protein-dependent signaling in the Hh pathway remains controversial (Ayers and Therond, 2010; Jiang and Hui, 2008).

The parallel between Smo and GPCRs also extends to its regulation by G-protein-coupled receptor kinases (GRKs). GRKs phosphorylate activated GPCRs to promote Arrestin-dependent receptor internalization, effectively shutting off G-protein signaling, in a process called homologous desensitization (Reiter and Lefkowitz, 2006). GRKs play a conserved role in promoting Hh signaling (Chen et al., 2004; Chen et al., 2011; Meloni et al., 2006; Philipp et al., 2008). In flies, the *Drosophila* ortholog of mammalian GRK4/5/6, called Gprk2, promotes Smo phosphorylation, internalization, and downregulation in Hh-responding cells. Loss-of-function mutations in *gprk2* impair high-threshold Hh target gene expression in wing imaginal discs, the epithelial precursor to the adult wing (Chen et al., 2010; Cheng et al., 2010; Molnar et al., 2007).

It is somewhat surprising that GRKs are required to promote target gene expression downstream of Smo. As these kinases typically function to limit GPCR signaling, their loss often leads to receptor hypersensitivity and too much activity rather than too little (Premont and Gainetdinov, 2007). Furthermore, strong accumulation of Smo at the cell surface, as is observed in *gprk2*-mutant Hh-responding

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cells (Cheng et al., 2010), is usually correlated with high-level signaling (Denef et al., 2000; Zhu et al., 2003). To explain the positive role of GRKs in Hh signaling, it has been suggested that they may be doing something more than just downregulating Smo. For example, there is evidence that GRKs can directly regulate the activation state of Smo (Chen et al., 2010; Chen et al., 2011). Misregulation of G-protein signaling and second messenger production in GRK mutants could also contribute to signaling impairment (Cheng et al., 2010). To distinguish between these and other possibilities, we set out to assess how GRK activity affects signaling downstream of Smo by analyzing the activity of canonical and G-protein-dependent signaling outputs in *gprk2* mutant flies.

MATERIALS AND METHODS

Fly strains and reagents

Fly strains and their sources: *UAS-smo*^{RNAi}, *UAS-sufu*^{RNAi} (transformants 9542 and 35055; Vienna Drosophila RNAi Center); *hh*-GAL4, *UAS-Ptc* (S. M. Cohen, Institute of Molecular and Cell Biology, Singapore); *UAS-mC**, *UAS-R** (D. Kalderon, Columbia University, New York, USA); *UAS-G*α_i^{Q205L} (J. Knoblich, Institute of Molecular Biotechnology, Vienna, Austria); *UAS-Smo*^{SD123}*-CFP* (J. Jiang, University of Texas Southwestern Medical Center, Dallas, TX, USA); *gprk2*^{del1}, *gprk2*^{del2}, *gprk2*^{KO} and *UAS-Gprk2* (Cheng et al., 2010). All other strains were from the Bloomington Drosophila Stock Center.

Primary antibodies and their sources: guinea pig anti-Gprk2 (Cheng et al., 2010); mouse anti-Col (M. Crozatier, Centre de Biologie du Développement, Toulouse, France), anti-myc (9E10; Santa Cruz Biotechnology), anti-α-Tubulin (12G10), anti-En (4D9), anti-Ptc (Apa I), anti-SuFu (25H3), and anti-Smo (20C6) (Developmental Studies Hybridoma Bank); rabbit anti-Gα_i (J. Knoblich), anti-Fu and anti-Cos2 (P. Therond, Université Nice, France), anti-GFP (Torrey Pines Scientific); rat anti-Smo (S. Cohen), anti-Ci^{155} (T. Kornberg, University of California, San Francisco, USA).

To prepare the EPAC-BRET plasmid, a fragment encoding the cAMP binding portion of human Epac1 (amino acids 148-881) fused to GFP10 and *Renilla* luciferase at the N- and C-termini, respectively (provided by Michel Bouvier, Université de Montréal), was subcloned into *pMT.puro* to generate *pMT.puro/GFP10-EPAC-RLucII_T781A,F782A*. As a control for background emission, we prepared a construct encoding the same protein lacking the GFP10 moiety. Gα_s^{Q215L}, Gα_i^{Q205L} and mC* coding sequences were PCR amplified from genomic DNA from flies bearing appropriate UAS-transgenes (Connolly et al., 1996; Li et al., 1995; Schaefer et al., 2001) and subcloned into *pMT.puro*. The four GPSA point mutations were generated by PCR mutagenesis in a *pMT.puro* construct encoding Smo fused to C-terminal GFP.

Tissue analyses

Immunostainings, wing mounting and measurements, and image analysis were performed as described (Cheng et al., 2010).

Cell culture analyses

gprk2 and *smo* dsRNA templates were prepared by PCR amplification of genomic DNA from *w*^{1118} flies. β-galactosidase-encoding (*lacZ*) dsRNA was used as a control. Cell culture, dsRNA preparation and treatments, and transfections were performed as described (Cheng et al., 2010). For experiments involving treatment with more than one dsRNA, the total amount of RNA added to cells was equalized with the use of *lacZ* dsRNA. In some experiments, cells were treated with control or Hh^N-conditioned medium for 1 to 2 hours before lysis, as previously described (Cheng et al., 2010).

Leptomycin B treatment of discs

Larval anterior halves with attached imaginal discs were dissected in serum-free insect medium (SFM) (Sigma) and transferred into SFM containing 50 ng/ml Leptomycin B (LMB) (Sigma). After incubation for 60 minutes at 29°C, samples were rinsed with PBS and then fixed and immunostained as above.

cAMP measurements

For bioluminescence resonance energy transfer (BRET) experiments, dsRNA-treated S2 cells were transiently transfected with *pMT.puro/GFP10-EPAC-RLucII_T781A,F782A*, encoding the EPAC-BRET cAMP sensor protein. In the unbound state, the GFP10 and *R*LucII moieties of this protein are in proximity such that the energy generated by *R*LucII after oxidizing its substrate coelenterazine (DeepBlueC) is transferred to GFP10, causing it to fluoresce. cAMP binding to the EPAC1 domain induces a conformational shift that decreases this intramolecular BRET (Jiang et al., 2007). Forty-eight hours after induction of sensor expression, cells were harvested, washed once with PBS, and transferred in PBS to white-walled, clear-bottomed 96-well plates. DeepBlueC (Biotium) was added at a final concentration of 5 μM and 1–4 minutes later emissions at 400 nm (*R*LucII, donor) and 515 nm (GFP10, acceptor) were measured using a PHERAstar microplate reader (BMG Labtech). The BRET signal was calculated as the ratio of GFP10: *R*LucII emission. The values were corrected for background emission by subtracting the BRET signal obtained using S2 cells transfected in parallel with a biosensor protein that lacks the GFP10 moiety, to yield the net BRET signal. Graphs represent the composite results from two independent experiments, each with triplicate or quadruplicate measurements.

For larval analyses, freshly hatched larvae from 4-hour embryo collections were transferred to food vials (40 per vial). Larvae were cultured at 29°C for 4 days. cAMP concentrations for six independent groups of eight wandering third-instar larvae of each genotype were measured using the CatchPoint cAMP 384-well Bulk Fluorescent Assay Kit (Molecular Devices). Briefly, eight larvae were homogenized in 300 μl of CatchPoint lysis buffer, and lysates were snap-frozen. After thawing, lysates were spun twice at 18,000 ***g*** and 4°C in a microcentrifuge and insoluble material and fat were removed. Measurements of cAMP in the soluble lysates were made according to the manufacturer's instructions. cAMP measurements were normalized to protein concentrations in the lysates as determined by DC Protein Assay (BioRad). Protein concentrations varied by less than 11% between genotypes.

RESULTS

Loss-of-function mutations in *gprk2* act in a temperature-dependent manner. Expression of several high-threshold targets is strongly impaired or lost at the restrictive temperature of 29°C, whereas the expression domain of the low-threshold target *decapentaplegic* (*dpp*) is expanded (Chen et al., 2010; Cheng et al., 2010; Molnar et al., 2007). As Smo is believed to be the principal target of Gprk2 in the pathway, we set out to assess how loss of Gprk2 affects signaling downstream of Smo, beginning with Ci regulation. Hh pathway activity is normally reflected in the pattern of accumulation of Ci in wing discs. Hh signaling stabilizes full-length Ci^{155} in a broad stripe of anterior compartment cells (detected using a Ci^{155}-specific antibody; Fig. 1A). Ci^{155} levels taper off anteriorly as Hh levels decrease and Ci gets proteolytically processed to Ci^{Rep}. In the first few rows of anterior cells abutting the anterioposterior (A/P) compartment boundary, Ci^{155} levels are also low as high-level signaling induces expression of Roadkill, which targets active Ci^{155} for ubiquitylation and degradation (Zhang et al., 2006).

In *gprk2* null mutants at the restrictive temperature Ci^{155} levels were uniformly high in a broader-than-normal stripe (Fig. 1B), as previously observed (Chen et al., 2010; Molnar et al., 2007). In anterior cells abutting the A/P boundary, the normal reduction in Ci^{155} levels was not observed, consistent with the presumed loss of high-threshold *roadkill* expression. Expression of a *gprk2* transgene using the dorsal compartment-specific *ap-GAL4* driver restored both the profile of Ci^{155} accumulation (Fig. 1C) and high-threshold target gene expression (Cheng et al., 2010) to normal in dorsal *gprk2* mutant cells, confirming the specificity of the effects. Smo accumulates abnormally in *gprk2*-mutant Hh-responding cells (Fig.

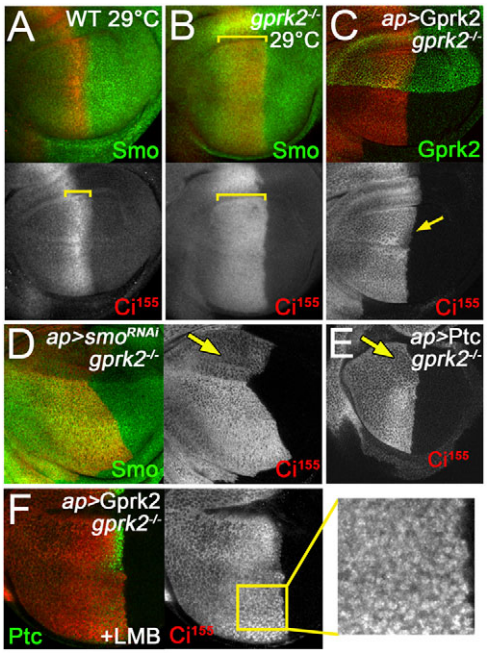

Fig. 1. Regulation of Ci in *gprk2* mutants. Discs in all figures are oriented with posterior to the right and dorsal side up. (**A**,**B**) Wild-type (A) and *gprk2* mutant (B) wing discs (29°C) stained for Smo (green) and Ci^{155} (red). (**C**) *gprk2* mutant wing disc (29°C) expressing a *gprk2* transgene in the dorsal compartment, stained for Gprk2 (green) and Ci^{155} (red). The arrow indicates a reduction of Ci^{155} in rescued cells adjacent to the A/P boundary. (**D**,**E**) *gprk2* mutant wing discs (29°C) with dorsal expression of *smo* dsRNA (D) or Ptc (E), stained for Smo (green) and Ci^{155} (red). The arrow indicates reversal of Ci^{155} stabilization (arrows). (**F**) 'Dorsal-rescued' *gprk2* mutant wing disc treated with LMB, stained for Ptc (green) and Ci^{155} (red). Nuclear Ci accumulation in *gprk2* mutant ventral cells is shown in zoom. Genotypes: w^{1118} (A); $gprk2^{del2}/gprk2^{KO}$ (B); *ap-GAL4/+;*$gprk2^{del2}$*,UAS-Gprk2/*$gprk2^{KO}$ (C,F); *ap-GAL4/+;*$gprk2^{del1}$*,UAS-*Smo^{RNAi}*/*$gprk2^{KO}$ (D); *ap-GAL4/+;*$gprk2^{del1}$*,UAS-Ptc/*$gprk2^{KO}$ (E).

1B). The domain of Ci^{155} stabilization coincided precisely with ectopic Smo, suggesting that the accumulated Smo protein was active. To confirm this, we inhibited Smo activity by expressing Ptc or by dsRNA-mediated depletion of Smo in dorsal compartment cells. Both manipulations abolished Ci stabilization (Fig. 1D,E). We conclude that Hh activates signaling through Smo to inhibit processing of Ci^{155} to Ci^{Rep} in *gprk2* mutant cells.

The *gprk2* mutant phenotypes (Ci^{155} stabilization, expansion of low-threshold targets, loss of high-threshold targets) are very similar to those observed in *fu* mutants, in which canonical signaling is blocked (Alves et al., 1998; Ohlmeyer and Kalderon, 1998). The inability of accumulated Ci^{155} to activate high-threshold target gene expression in *fu* mutants is largely owing to a failure to inactivate SuFu, which retains Ci^{155} in the cytoplasm (Methot and Basler, 2000). Because of the similarities with *fu* mutants, we tested whether SuFu-dependent cytoplasmic retention could explain the loss of target gene expression in *gprk2* mutants. dsRNA-mediated depletion of SuFu partially rescued *ptc* expression in a *fu* mutant background, as expected, but had no effect in *gprk2* mutants (supplementary material Fig. S1A-C), supporting the argument against this possibility. In fact, in $gprk2^{-/-}$*,ap>Gprk2* wing discs incubated with the nuclear export inhibitor LMB (Wang et al., 2000), nuclear accumulation of Ci^{155} was observed both in *gprk2* mutant ventral and 'rescued' dorsal compartment Hh-responding cells (Fig. 1F). This effect was observed with multiple *gprk2* alleles, and differed from *fu* mutants, which failed to accumulate nuclear Ci^{155} under these conditions (supplementary material Fig. S1D-H). We conclude that Smo activity is sufficient to promote both stabilization and nuclear entry of Ci^{155} in the absence of Gprk2. However, high-threshold target genes are not expressed, perhaps because of a failure to fully activate Ci^{155} (Chen et al., 1999).

Gprk2 limits activation of the canonical pathway by Smo

The analysis of Ci regulation suggested that Smo retains significant ability to activate canonical signaling in the absence of Gprk2. To compare, at a molecular level, the extent of canonical pathway activation by Smo in the presence and absence of Gprk2, we examined the phosphorylation status of Fu, Cos2 and SuFu in wild-type and *gprk2* mutant wing discs at the restrictive temperature. Phosphorylation of these three proteins provides a direct readout of Smo activity (Lum et al., 2003; Nybakken et al., 2002). In wing discs, most Smo activity derives from the posterior compartment, where the pathway is constitutively active (Denef et al., 2000). Gprk2 does not effectively regulate Smo in posterior cells, probably because it is not transcriptionally upregulated in these cells as it is in Hh-responding anterior cells (Chen et al., 2010; Cheng et al., 2010). Therefore, in order to limit our analysis to anterior cells, we eliminated constitutive pathway activity in the posterior compartment by expressing *smo* dsRNA under the control of *hh*-GAL4 (supplementary material Fig. S2A,B). In immunoblots of control *hh*-GAL4>smo^{dsRNA} disc lysates, Smo protein from wild-type anterior compartment cells appeared as a smear of at least two distinct forms (Fig. 2A). In *gprk2* mutants, a 2.2-fold increased level of a faster migrating form of Smo was detected, consistent with the previously observed ectopic accumulation of hypophosphorylated Smo (Cheng et al., 2010). To our surprise, the levels of phosphorylation of downstream components of the pathway were markedly increased in *gprk2* mutant discs compared to controls. The phosphorylated forms of Fu, Cos2 and Sufu were 1.8-, 2.1- and 4.2-fold more abundant in the absence of *gprk2*, respectively. Ci^{155} levels were also 2.9-fold higher in the mutants. These increases were all in a similar range to the increase in Smo protein levels, suggesting that signaling from Smo to the downstream components is not dramatically impaired by the loss of Gprk2.

Because Ptc binds to Hh and promotes its internalization, failure to induce *ptc* expression in wing discs leads to a broadening of the Hh gradient (Chen and Struhl, 1996). The observed increase in pathway activity in *gprk2* mutants could thus be due simply to more cells being exposed to Hh. To rule out this possibility, we turned to a simpler cell culture model. Under our standard culture conditions Smo exists in a partially phosphorylated state in S2 cells (supplementary material Fig. S2C), indicating that there is a basal level of Smo activation (as previously observed with cl8 cells) (Denef et al., 2000). Stimulation of control dsRNA-treated S2 cells with Hh^{N}-conditioned medium induced phosphorylation of Fu, Cos2 and SuFu, as expected (Fig. 2B). dsRNA-mediated depletion of Gprk2, which causes a substantial increase in cell-surface Smo levels in S2 cells (Cheng et al., 2010), was on its own sufficient to induce phosphorylation of downstream pathway components (Fig. 2B). This was most likely owing to enhancement of basal Smo activity, as simultaneous depletion of Smo abolished Fu and Cos2

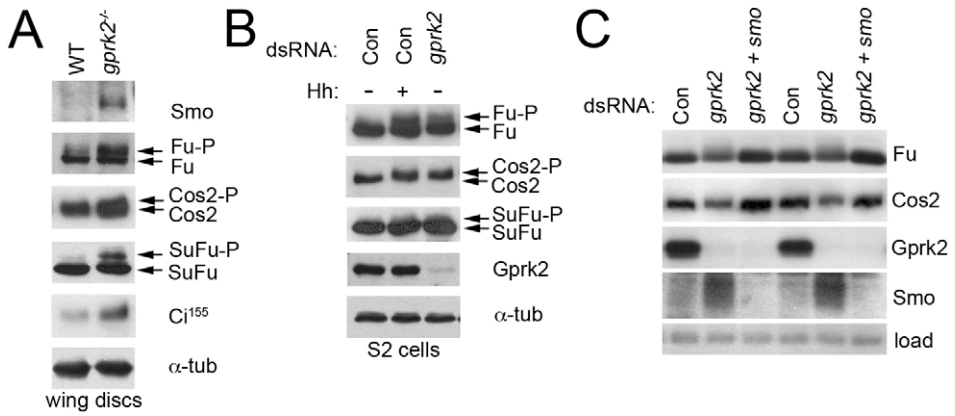

Fig. 2. Gprk2 inhibits canonical pathway activation. (**A**) Western blot analysis of wing disc lysates from larvae cultured at 29°C. Compared to controls, *gprk2* mutants show higher levels of Smo, as well as increases in full-length Ci^{155} and the slower-migrating, phosphorylated forms of Fu, Cos2 and SuFu. α-tubulin served as a loading control. (**B**) Western blot analysis of S2 cell lysates. Hh-treatment of control S2 cells and *gprk2* depletion alone both induced phosphorylation of components of the downstream signaling pathway. α-tubulin served as a loading control. (**C**) Western blot analysis of S2 cell lysates, analyzed in duplicate samples. The increase in Fu and Cos2 phosphorylation induced by *gprk2* depletion was eliminated by simultaneous depletion of Smo. A background band was used to ensure equal loading (load). Genotypes: *UAS-Smo^{RNAi}/hh-GAL4* (WT control) and *gprk2^{del1},UAS-Smo^{RNAi}/gprk2^{KO},hh-GAL4* (*gprk2$^{-/-}$*) (A).

phosphorylation (Fig. 2C). Similarly, depletion of Gprk2 enhanced the level of canonical pathway activation in cells treated with non-saturating levels of Hh^N (supplementary material Fig. S3). We conclude that Smo is active in the absence of Gprk2 and signals through the canonical pathway to promote Ci^{155} stabilization and nuclear entry. The observed increase in canonical pathway activity in vivo and in cells is consistent with Gprk2 functioning in a manner typical of GRKs to induce activity-dependent turnover of Smo and terminate its activity. However, it is not obvious why Hh target gene expression would be lost in this case.

Elimination of Gprk2 lowers basal cellular cAMP levels

In the absence of obvious signs of canonical signaling impairment, we next tested whether G-protein-dependent signaling is altered in *gprk2* mutants. It is well established that elimination of GRKs or of their target sites in a given receptor can have major effects on the levels and dynamics of heterotrimeric G-protein signaling and second messenger production in response to GPCR activation (Kavelaars et al., 2003; Peppel et al., 1997; Ren et al., 2005; Violin et al., 2006; Wang et al., 2009). Given the central role of Pka in Hh signaling, cAMP misregulation could have significant effects on target gene expression. Consistent with a role for Gprk2 in cAMP regulation, baseline ovarian cAMP levels were about threefold lower than normal in flies homozygous for a tissue-specific regulatory mutant allele of *gprk2* (Lannutti and Schneider, 2001). We made similar observations in *gprk2* null mutants during developmental stages. Compared with age-matched wild-type controls, cAMP levels were 60% lower in *gprk2^{del1}/gprk2^{del1}* and *gprk2^{del1}/gprk2^{del2}* wandering third-instar larvae (Fig. 3A). To see if this reflected a reduction in cAMP concentration at a cellular level rather than a more central effect, we looked at the effects of Gprk2 depletion in S2 cells expressing a BRET biosensor for cAMP, called EPAC-BRET (Jiang et al., 2007). We first confirmed that this biosensor was capable of detecting both increases and decreases in cAMP levels in S2 cells (supplementary material Fig. S4A). Compared to control dsRNA, treatment of S2 cells with *gprk2* dsRNA caused a significant increase in net BRET (*t*-test, $P<0.005$) (Fig. 3B), indicating that Gprk2 depletion reduced basal cellular cAMP levels. A similar effect was observed with independent dsRNAs targeting *gprk2* non-coding sequences (supplementary material Fig. S4B), excluding the possibility of off-target effects. Thus in both animals and cultured cells, elimination of Gprk2 reduces cAMP levels. This could be as a result of

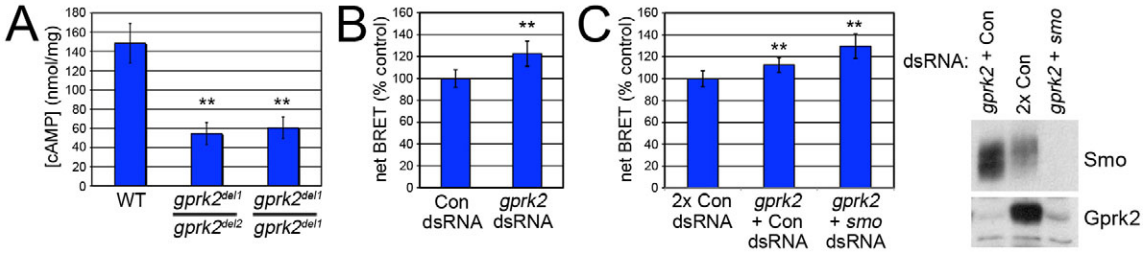

Fig. 3. Loss of Gprk2 reduces cAMP levels. (**A**) Compared with age-matched controls, cAMP levels were ~60% lower than normal in two different genotypes of *gprk2* mutant larvae (29°C) (*t*-test versus control: **, $P<0.005$). (**B**,**C**) Measurement of cellular cAMP levels by BRET in EPAC-BRET-expressing S2 cells. An increase in net BRET ratio indicates a reduction in cAMP levels. Treatment of cells with dsRNA targeting *gprk2* significantly lowered cAMP levels (*t*-test versus *lacZ* dsRNA treatment control: **, $P<0.005$) (B). Simultaneous depletion of Smo and Gprk2 did not counteract the effects of Gprk2 depletion alone on cAMP levels (*t*-test versus *lacZ* dsRNA treatment control: **, $P<0.005$). Western blot at right demonstrates knockdown efficiency. (C). Genotypes: *w^{1118}*, *gprk2^{del1}/gprk2^{del1}* and *gprk2^{del1}/gprk2^{del2}* (A).

misregulation of G-protein-dependent signaling by Smo itself (Ogden et al., 2008). However, unlike canonical pathway activation (Fig. 2C) or Ci stabilization (Fig. 1C), the effect of eliminating Gprk2 on cAMP levels was not reversed by simultaneous depletion of Smo (Fig. 3C). Thus the reduction in cAMP levels is not only attributable to Smo activity, but may involve misregulation of other GPCRs as well.

Low levels of cAMP impair Hh signaling in *gprk2* mutants

If reduced cAMP levels contribute to Hh signaling impairment in *gprk2* mutants, one might expect a further reduction to cause more severe impairment of target gene expression, whereas an increase should rescue it. We tested this hypothesis in *gprk2* mutant discs. To lower cAMP levels, we expressed $G\alpha_i^{Q205L}$, a constitutively active mutant form of $G\alpha_i$ that inhibits adenylate cyclase (Schaefer et al., 2001), in the dorsal compartment. These experiments were carried out at permissive temperature (27°C), at which Hh pathway activity is just sufficient for normal wing patterning in the absence of Gprk2 (Cheng et al., 2010). Although it ectopically activated expression of a low-threshold *dpp* reporter (Ogden et al., 2008), expression of $G\alpha_i^{Q205L}$ in wild-type animals had little effect on high [anterior Engrailed (En), Collier (Col; Knot – FlyBase)] or intermediate (Ptc) threshold target expression (Fig. 4A-C). However, in the absence of Gprk2, $G\alpha_i^{Q205L}$ strongly suppressed expression of all three (Fig. 4D-F). Thus *gprk2* mutant Hh-responding cells are sensitive to reduction of cAMP production in a way that wild-type cells are not. By comparison, $G\alpha_i^{Q205L}$ expression did not suppress Ptc expression in *fu* mutants under the same conditions (supplementary material Fig. S5A,B), indicating that this is not a general effect. cAMP levels are limiting for Hh signaling specifically in the absence of Gprk2.

If cAMP levels are limiting, then stimulating cAMP production should restore Hh target gene expression in *gprk2* mutants. To test this, we expressed an activated form of $G\alpha_s$ ($G\alpha_s^{Q215L}$) (Connolly et al., 1996), which stimulates adenylate cyclase, at restrictive temperature (29°C). In a wild-type background, expression of $G\alpha_s^{Q215L}$ had little effect on Ci accumulation or Hh target gene expression (Fig. 4G-I). In the absence of Gprk2, however, $G\alpha_s^{Q215L}$ expression restored target gene activation in Hh-responding cells (Fig. 4J-L). Even expression of the highest threshold target, anterior *en*, was rescued (Fig. 4L). This was accompanied by normalization of Ci^{155} accumulation (Fig. 4J). There was no ectopic pathway activation, indicating that rescue of target gene expression was dependent on activation of the endogenous signaling pathway by Hh and Smo. The effect was again specific, as $G\alpha_s^{Q215L}$ expression did not rescue target gene expression in *fu* mutants (supplementary material Fig. S5C,D). Taken together, our results indicate that cAMP levels are low and limiting for pathway activation in the *gprk2* mutants.

Increased Pka activity restores Hh target gene expression in *gprk2* mutants

Given that Pka is one of the main cellular targets of cAMP, we next asked whether Pka activity is limiting for Hh target gene expression in the mutants. If so, inhibiting Pka activity would be expected to further impair target gene expression, whereas activating Pka should restore it. As a first indication that Pka activity is indeed limiting, we observed strong genetic interactions between alleles of *gprk2* and *pka-c1* (encoding the principal Pka catalytic subunit involved in Hh signaling) (Jiang and Struhl, 1995; Li et al., 1995). Hh signaling patterns the central region of the adult wing bounded by wing veins L3 and L4, and reductions in Hh signaling activity lead to progressive decreases in L3-L4 intervein area. In wings from *pka-c1* heterozygous flies raised at 27°C, L3-L4 intervein area as a fraction of total wing area was indistinguishable from wild type (Fig. 5A,B,E). In *gprk2* mutants, a just-perceptible but statistically significant reduction was observed (*t*-test versus wild type, $P<0.001$) (Fig. 5C,E). However, *gprk2* null, *pka-c1* heterozygous animals showed a marked reduction in L3-L4 area (*t*-test versus *gprk2* null, $P<0.001$) (Fig. 5D,E). This indicates that lowering endogenous Pka levels reduced Hh signaling activity in the absence of Gprk2. To test this further, we directly inhibited Pka activity in wing discs by expressing PKA-R*, a cAMP-insensitive form of the regulatory subunit, at the permissive temperature of 27°C. Consistent with previous reports (Li et al., 1995), PKA-R* expression stabilized Ci and induced Hh-independent ectopic expression of Col and Ptc throughout the anterior compartment of otherwise wild-type discs (Fig. 5F,G). The level of activation was lower than that observed in endogenous Hh-responding cells. In a *gprk2* mutant background, expression of PKA-R* still induced low-level ectopic Col and Ptc expression (Fig. 5H,I). However, it also completely suppressed endogenous expression of Col and Ptc. The selective sensitivity of *gprk2* mutant Hh-responding cells to Pka inhibition is consistent with Pka activity being limiting for functioning of the pathway.

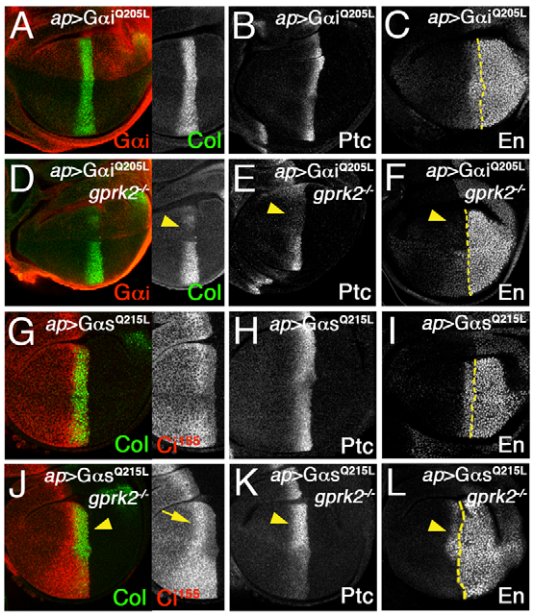

Fig. 4. cAMP is limiting for Hh target gene expression in the absence of Gprk2. (**A-F**) Expression of activated $G\alpha_i$ ($G\alpha_i^{Q205L}$) (red in A and D) in the dorsal compartment of wild-type (A-C) and *gprk2* mutant (D-F) wing discs (27°C). In wild-type discs, expression of Col (A), Ptc (B) and En (C) was unaffected by $G\alpha_i^{Q205L}$. In *gprk2* mutant discs, expression of Col (D), Ptc (E) and En (F) was inhibited by increased $G\alpha_i$ activity (arrowheads). (**G-L**) Expression of activated $G\alpha_s$ ($G\alpha_s^{Q215L}$) in the dorsal compartment of wild-type (G-I) and *gprk2* mutant (*J-L*) wing discs (29°C). In wild-type discs, expression of Col (G), Ptc (H) and En (I) was unaffected by $G\alpha_s^{Q215L}$. In *gprk2* mutant discs, $G\alpha_s$ activation restored Ci^{155} accumulation (red) to normal (arrow, J), and expression of Col (green) (J), Ptc (K) and En (L) was specifically rescued in Hh-responding cells (arrowheads). Genotypes: *ap-GAL4/+; UAS-*$G\alpha_i^{Q205L}$*/+* (A-C); *ap-GAL4/+;gprk2*^{del1}*,UAS-*$G\alpha_i^{Q205L}$*/gprk2*^{KO} (D-F); *ap-GAL4/+;UAS-*$G\alpha_s^{Q215L}$*/+* (G-I); *ap-GAL4/+;gprk2*^{del1}*,UAS-*$G\alpha_s^{Q215L}$*/gprk2*^{KO} (J-L).

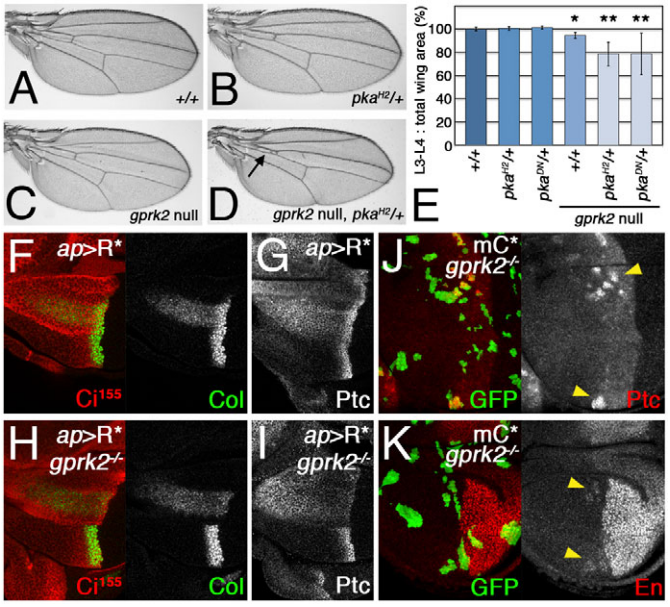

Fig. 5. PKA activity is limiting for Hh target gene expression in the absence of Gprk2. (**A-E**) The ratio of L3-L4 to total wing area was measured for wings from wild-type (A), *pka-c1*^{H2} heterozygous (B), *gprk2* null (C) and *gprk2* null, *pka-c1*^{H2} heterozygous (D) flies, cultured at 27°C. Averages based on the measurement of 11 to 19 wings of each genotype are plotted in (E). *t*-test versus wild type: *, $P<0.001$; *t*-test versus *gprk2* null: **, $P<0.001$. (**F-I**) Expression of a non-regulatable Pka regulatory subunit, R*, in the dorsal compartment of wild-type (F-G) and *gprk2* mutant (*H-I*) wing discs (27°C). Reduction of Pka activity blocked endogenous expression of Col (green) (*H*) and Ptc (*I*) in *gprk2* mutants. (**J**,**K**) Expression of constitutively active Pka catalytic subunit, mC*, in clones of cells marked by co-expression of GFP (green) in *gprk2* mutant wing discs (29°C). mC* rescued expression of Ptc (red) (*J*) and En (red) (*K*) in more-proximal anterior clones located within the range of endogenous Hh (arrowheads). Genotypes: w^{1118} (+/+) (A); *pka-c1*^{H2}/+ (B); *gprk2*^{del1}/*gprk2*^{KO} (*gprk2* null) (C); *pka-c1*^{H2}/+;*gprk2*^{del1}/*gprk2*^{KO} (D); in addition, *pka-c1*^{DN}/+ and *pka-c1*^{DN}/+;*gprk2*^{del1}/*gprk2*^{KO} wings were included in measurements in (E); *ap-GAL4*/+;*UAS-R**/+ (F,G); *ap-GAL4*/+;*gprk2*^{del1},*UAS-R**/*gprk2*^{KO} (H,I); *hs-FLP*/+;*act>>GAL4,UAS-GFP*/+;*UAS-mC**,*gprk2*^{del1}/*gprk2*^{KO} (J,K).

To confirm this, we expressed a constitutively activated form of a mouse catalytic subunit, mC* (Li et al., 1995). As *ap*-GAL4-driven mC* expression was lethal, we analyzed GFP-marked mC*-expressing clones generated in *gprk2* mutants raised at restrictive temperature. mC* had a position-dependent effect, restoring expression of Ptc and En specifically in proximal anterior cells within the range of endogenous Hh (Fig. 5J,K). More distal cells near the dorsoventral compartment boundary may be less responsive owing to attenuation of Hh signaling by Notch and Wingless in this region (Glise et al., 2002). Taken together, our results indicate that Gprk2 is required for the normal functioning of a positively acting, cAMP- and Pka-dependent component of the Hh signaling pathway.

Loss of Gprk2 affects Pka-dependent Smo phosphorylation and activity

Smo accumulates in a hypophosphorylated form in the absence of Gprk2, which was thought to reflect the loss of direct Gprk2-mediated phosphorylation (Cheng et al., 2010). A recent analysis suggested that Gprk2 does indeed play a direct role in regulating Smo, in two ways (Chen et al., 2010). According to the proposed model, phosphorylation of Smo by Gprk2 at four sites in its C-terminal cytoplasmic tail is required for Smo to adopt its most active conformation. Gprk2 binding and dimerization were also proposed to promote Smo activity by facilitating intermolecular association of Smo cytoplasmic tails. In our rescue experiments, increasing cAMP levels or Pka activity was sufficient to restore apparently normal functioning of the Hh pathway in the complete absence of Gprk2. This suggested that direct regulation of Smo by Gprk2 is not absolutely required for high-level Smo signaling. In fact, we noticed that phosphorylation of a form of Smo lacking all four Gprk2 phosphorylation sites (Smo^{GPSA}-GFP) (Chen et al., 2010) was reduced as a result of Gprk2 depletion to a similar extent to wild-type Smo in Hh^{N}-treated cells (Fig. 6A). This indicates that phosphorylation at these four sites represents only a fraction of the Gprk2-dependent changes in Smo phosphorylation under normal circumstances, and points to the possibility that Gprk2 could have indirect effects on Smo through another kinase.

Smo is the principal target of positive regulation by Pka in the Hh pathway. Pka phosphorylates Smo at three sites in its C-terminal cytoplasmic tail, with the level of phosphorylation correlating with Smo cell surface accumulation and activity (Apionishev et al., 2005; Jia et al., 2004; Zhang et al., 2004). By causing cAMP levels to drop, elimination of Gprk2 could indirectly impair Hh responsiveness by reducing Pka-dependent phosphorylation of Smo. In this case, the Smo phosphorylation shift observed in response to Gprk2 depletion could be due in part to reduced Pka and Pka-primed phosphorylation. Consistent with this, expression of mC* eliminated the difference in Smo mobility between control and Gprk2-depleted S2 cells stimulated with Hh^{N} (Fig. 6B). mC* expression did not noticeably reduce the mobility of the most phosphorylated form of Smo in control cells, indicating that it did not non-specifically phosphorylate Smo. These results suggest that the reduction in Smo phosphorylation seen in the absence of Gprk2 is due in large part to reduced Pka activity.

Pka regulates Smo conformation, with increased phosphorylation causing a greater conformational shift to a more active state (Zhao et al., 2007). Although we observed an increase in Smo levels and pathway activity in the absence of Gprk2, the Smo that accumulates may not be fully activated. To determine if the positive effects of cAMP and Pka uncovered in *gprk2* mutants can be attributed to increased phosphorylation of Smo, we tested the ability of a form of Smo that mimics phosphorylation at all three Pka/CkI clusters (Smo^{SD123}) (Jia et al., 2004) to restore high-threshold target gene expression. *ap*-GAL4-driven expression of Smo^{SD123} stabilized Ci^{155} and induced strong ectopic Ptc and En expression throughout the dorsal anterior compartment of wild-type discs, as expected (Fig. 6C,E). In a *gprk2* mutant background, Smo^{SD123} also stabilized Ci^{155} and restored expression of Ptc in endogenous Hh-responding cells, but ectopic Ptc expression was strongly reduced (Fig. 6D). Furthermore, no En expression was induced in dorsal-anterior cells (as previously observed by others) (Chen et al., 2010; Molnar et al., 2007) (Fig. 6F). The fact that Smo^{SD123} expression can induce some target gene expression in the mutants confirms that part of the signaling defect in *gprk2* mutants is due to reduced Pka-dependent phosphorylation of Smo. However, increasing cAMP production or Pka activity efficiently induces En expression in the absence of Gprk2, whereas mimicking Pka phosphorylation of Smo does not. This suggests that the positive effects of Pka are mediated at least in part by a target other than the three identified phosphorylation sites in Smo, similar to that observed previously in *Drosophila* embryos (Zhou et al., 2006).

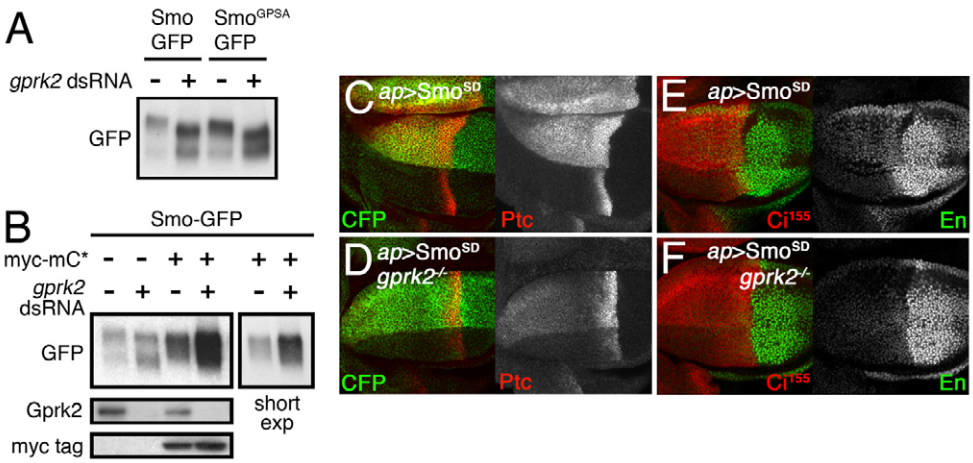

Fig. 6. The positive effects of Pka in *gprk2* mutants are mediated in part through Smo phosphorylation. (**A**) Western blot analysis of lysates from S2 cell transfected with Smo-GFP or Smo^{GPSA}-GFP, treated with control (–) or *gprk2* (+) dsRNA, and then stimulated with Hh^{N}-conditioned medium for 2 hours. Smo^{GPSA} lacking Gprk2 phosphorylation sites showed a phosphoshift in response to Gprk2 depletion. (**B**) Western blot analysis of lysates from control and myc-tagged mC*-transfected S2 cells, treated with control (–) or *gprk2* (+) dsRNA, and then stimulated with Hh^{N}. mC* expression essentially eliminated the Smo phosphoshift in Gprk2 depleted cells compared to control cells. Panel at right shows a shorter exposure of the mC*-expressing samples. (**C-F**) Expression of Smo^{SD123}-CFP protein in the dorsal compartment of wild-type (C,E) and *gprk2* mutant (D,F) wing discs (29°C). Smo^{SD123}-CFP and Ptc are in green and red, respectively in C and D. En and Ci^{155} are in green and red, respectively in E and F. Smo^{SD123} was less active in a *gprk2* mutant background. Genotypes: *ap-GAL4/+;UAS-Smo^{SD123}-CFP/+* (C,E); *ap-GAL4/+;gprk2^{del1},UAS-Smo^{SD123}-CFP/gprk2^{KO}* (D,F).

DISCUSSION

Based on existing evidence, the role of GRKs in Hh signaling is complex. Elimination of Gprk2 in flies leads to increased accumulation of Smo at the cell surface, generally a sign of high-level signaling and consistent with the typical function of GRKs in negatively regulating GPCRs. However, activation of Hh target genes is lost, indicating that Gprk2 plays a positive role in the pathway. Based on our analysis of how signaling downstream of Smo is affected in the absence of Gprk2, we draw three main conclusions that provide insight into this apparent contradiction (see model, Fig. 7). First, Gprk2 does indeed act as a negative regulator of the Hh pathway by limiting accumulation of active Smo. Second, Gprk2 activity is required for normal regulation of cellular cAMP levels, and thus Pka activity. Third, much of the positive effect of Gprk2 on Hh pathway activation is indirect, through promotion of Pka-dependent Smo phosphorylation and activation. Each of these conclusions is discussed below.

Gprk2 as a negative regulator of Hh signaling

GRKs regulate homologous desensitization of GPCRs, thereby limiting their signaling activity. Consequently, GRK loss often leads to increased surface receptor levels and exaggerated signaling responses (Claing et al., 2002; Premont and Gainetdinov, 2007). Consistent with this, we previously showed that Gprk2 downregulates Smo subsequent to its activation (Cheng et al., 2010). Here, we find that Smo-dependent activation of the canonical Hh pathway is increased at a cellular level in the absence of Gprk2, in a manner roughly proportional to the increase in Smo levels. This is the first demonstration that Gprk2 does in fact restrict Smo activity. Taken together the evidence suggests that Smo undergoes Gprk2-dependent homologous desensitization; that in the absence of Gprk2, Smo activity is not dramatically impaired; and that the accumulation of active Smo leads to more total pathway activation. As a result, Ci^{155} is stabilized and enters the nucleus in *gprk2*-mutant Hh-responding cells, as in wild-type cells. However, high-threshold target genes are not expressed.

Some of the same features of Hh pathway misregulation are found in other pathway mutants. Loss of Fu leads to similar effects on Ci stabilization and target gene expression (Alves et al., 1998). In contrast to *fu* mutants, SuFu is strongly phosphorylated and Ci^{155} accumulates in the nucleus in *gprk2* mutants. Manipulation of SuFu and Gα activity clearly points to distinct underlying causes of the signaling defects in these mutants. In *dally* and *lipophorin* (*lpp*; *Rfabg* – FlyBase) mutants, Ci^{155} stabilization and nuclear import are uncoupled from target gene expression, as in the *gprk2* mutants (Eugster et al., 2007; Khaliullina et al., 2009). Knockdown of *lpp* lipoprotein particle production in particular has some similar, if more severe, consequences as loss of *gprk2* – ectopic Smo stabilization and nuclear accumulation of Ci^{155} (although in cells *not* exposed to Hh), without activating target genes. Although the phenotypic similarities suggest that Gprk2 could work through the same mechanism, there are important differences that suggest this is unlikely. For example, in contrast to *gprk2* mutants, high-threshold target genes are expressed in the absence of Dally and Lpp, although over a narrower range (Eugster et al., 2007; Khaliullina et al., 2009). Loss of *lpp* appears to impair the ability of Ptc to silence Smo in the absence of Hh (Khaliullina et al., 2009), whereas Ptc regulates Smo normally in the absence of Gprk2 (Cheng et al., 2010). Furthermore, the genetic and biochemical evidence for Gprk2 acting directly on Smo is compelling (Chen et al., 2010). Instead of a direct mechanistic link, what these mutants may have in common is accumulation of a not-fully active form of Smo. In *lpp* and *dally* mutants, this is due to inappropriate Smo activation in the absence of Hh, whereas in *gprk2* mutants it reflects the failure to downregulate Smo in Hh-responding cells.

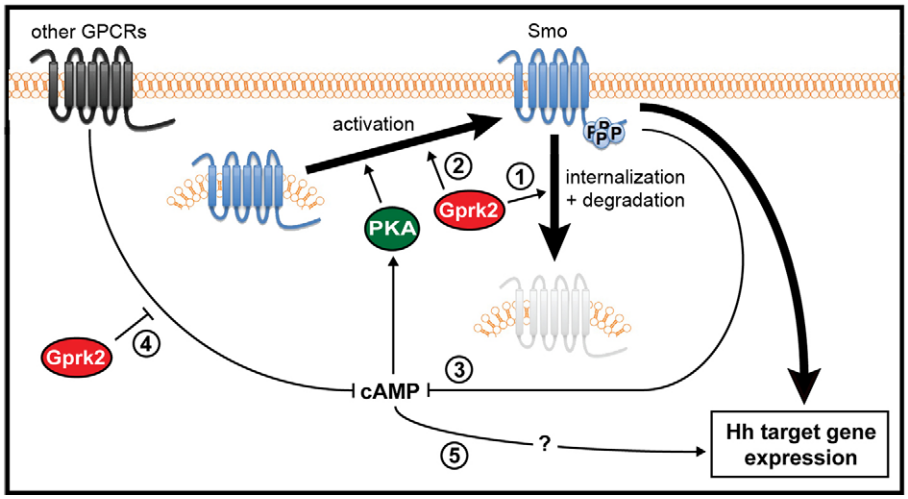

Fig. 7. Model for positive and negative regulation of Hh signaling by Gprk2. Upon exposure of cells to Hh, Smo is phosphorylated by Pka and activated, and accumulates at the plasma membrane, where it signals to activate target gene expression. On the one hand, Gprk2 negatively regulates Smo by phosphorylating it to promote its internalization and degradation (1). On the other hand, Gprk2 plays a positive role in promoting Hh target gene expression. This is due, in part, to an effect of direct Gprk2 binding and phosphorylation in activating Smo (2). It also reflects an indirect function of Gprk2 in maintaining cAMP levels in cells, perhaps by downregulating the levels of Smo and other GPCRs, thereby limiting signaling through Gαi [(3) and (4)]. In the absence of Gprk2, abnormally low cAMP levels lead to decreased Pka activity, incomplete Smo activation, and a loss of high-threshold target gene expression. It may also affect the activity of a distinct, positively acting Pka target in the Hh pathway (5).

Gprk2 is required for proper cAMP regulation

Extending the results of Lannutti et al. (Lannutti et al., 2001), we find that the reduction of cAMP levels caused by loss of Gprk2 is a general effect, occurring not only in adult animals but during development and in cultured cells as well. Importantly, our results clearly implicate this cAMP misregulation in the impairment of Hh target gene expression. The sensitivity of *gprk2* mutants to alterations in cAMP levels or Pka activity indicates that these are limiting for target gene expression in the mutants. This is not the case in *fu* mutants, highlighting the specificity of the effect.

An important outstanding issue is why cAMP levels decrease in *gprk2* mutants. Given that Smo can signal through $G\alpha_i$ (Ogden et al., 2008), excessive Smo-$G\alpha_i$ coupling (as observed for other GPCRs in GRK knockout mice) (Gainetdinov et al., 2003; Gainetdinov et al., 1999) would be the most obvious explanation. However, the fact that elimination of Smo in Gprk2-depleted S2 cells does not restore cAMP levels to normal means that there must be other factors. This is consistent with the magnitude of the drop in cAMP levels observed in *gprk2* mutant larvae, which would be difficult to explain solely in terms of Smo activity.

An alternative explanation is that misregulation of G-protein-dependent signaling by GPCRs other than Smo in the *gprk2* mutants affects cAMP levels and output of the Hh pathway. Individual GRKs can regulate many GPCRs and loss of a single GRK can have a major impact on cAMP production, as previously observed in GRK3 knockout mice (Peppel et al., 1997). Furthermore, cross-talk between the Sonic Hh pathway and Gα-coupled GPCRs has been demonstrated in mammals (Klein et al., 2001; Lelievre et al., 2008). A more global misregulation of Gprk2-regulated GPCRs could thus explain the changes in cAMP levels and impairment of Hh signaling seen in the absence of this kinase.

A positive role for Pka in Hh signaling

The *gprk2* mutants reveal an important positive role for Pka in Hh signaling. Both our genetic and biochemical analyses suggest that reduced Pka-dependent phosphorylation and activation of Smo contributes to the Hh signaling defect in *gprk2* mutants. How can we reconcile the seemingly contradictory observations that elimination of Gprk2 increases Smo-dependent canonical pathway activity while also reducing Smo activation? In the absence of Gprk2, activated Smo accumulates to ~3-fold higher levels than normal (Cheng et al., 2010). The increase in total canonical pathway activation can most easily be explained by the increase in Smo levels or duration of its activity. However, because of reduced Pka phosphorylation, individual Smo molecules may be unable to achieve the most active conformation required for full Ci activation.

If the positive effect of Pka were mediated entirely through the three phosphorylation sites in Smo, one would expect Smo^{SD123} to be at least as effective as $G\alpha_s$ or Pka in rescuing target gene expression in the *gprk2* mutants. That this seems not to be the case implies that a Pka target other than the three sites in Smo is required for maximal target gene activation. This is consistent with observations in other systems. Despite evidence for a positive role for Pka in vertebrate Hh signaling (Milenkovic et al., 2009; Tiecke et al., 2007), the three sites in the Smo C-terminus are not conserved and there is no clear evidence that Pka phosphorylates vertebrate Smo proteins (Ayers and Therond, 2010). Analyses in *Drosophila* embryos also point to the existence of a positively acting Pka target other than the three sites in Smo (Zhou et al., 2006). This target is unlikely to be Ci, as Pka strongly inhibits Ci^{155} both by promoting its cleavage and reducing its specific activity (Marks and Kalderon, 2011; Price and Kalderon, 1999). In fact, the stimulatory effects of Pka on Hh target gene transcription in embryos appear to be mediated by promoter sequences distinct from known Ci binding sites (Zhou et al., 2006).

Our conclusion that Gprk2 affects Hh signaling indirectly through Pka appears to contradict a recent study (Chen et al., 2010) that found that direct binding and phosphorylation of Smo by Gprk2 are required for high-level signaling. In fact, both mechanisms may normally contribute to Smo activation. Mutation of the Gprk2 sites at serine-740 and -741 (GPS1) to alanine

reduced the ability of overexpressed Smo^{SD123} to activate ectopic *en* expression, pointing to the importance of phosphorylation at these sites for maximal Smo activity (Chen et al., 2010). It may be that other kinases can substitute for Gprk2 to phosphorylate these sites in some circumstances. We previously showed that the second *Drosophila* GRK, Gprk1, does contribute to Hh pathway regulation at permissive temperature in the absence of Gprk2, making it a potential candidate (Cheng et al., 2010). However, unlike Gprk2, Gprk1 does not associate with Smo (Chen et al., 2010) nor have we been able to demonstrate direct regulation of Smo by this kinase (unpublished observations), suggesting that its effects may also be indirect. It is also possible that both Gprk2 and Pka can phosphorylate the GPS1 sites, or that Pka acting through a distinct positively acting target renders Hh target genes easier to activate, bypassing the requirement for GPS1 phosphorylation. In any case, it is clear from our results that the Gprk2-dependent changes in Smo phosphorylation extend well beyond the four mapped Gprk2 sites. The full effects, both direct and indirect, of Gprk2 on Smo and their respective contributions to Smo activation will need to be carefully defined.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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