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Grking the Smoothened Signal!

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One-Sentence Summary: Studies in flies and vertebrates reveal how the essential Hedgehog signaling component GRK2 functions at the level and downstream of Smoothened.

Abstract
G protein–coupled receptor (GPCR) kinase 2 (GRK2) has been implicated in a wide range of biological processes, including both activation and internalization of GPCRs, and is considered a potential therapeutic target for indications such as heart failure. This pleiotropic kinase has been linked to the clinically important Hedgehog (HH) signaling pathway, where it is paradoxically required for signal transduction yet also promotes internalization and degradation of the critical HH signal transducer Smoothened. Two reports in this issue of *Science Signaling* provide new insights into the role of GRK2 in HH signaling. Li *et al.* discovered that the *Drosophila melanogaster* homolog of GRK2, Gprk2, promotes Smoothened turnover through the Smurf family of E3 ligases. Pusapati *et al.* determined that mammalian GRK2 functions at the level of GPCRs downstream of Smoothened to set the overall sensitivity of cells to HH ligands.

The HH ligands are evolutionarily conserved morphogens that are critical for development. They bind to and block the activity of the membrane protein Patched (Ptc or PTCH), which relieves its repression of the G protein–coupled receptor (GPCR)-like protein Smoothened (Smo). Smo activation leads to changes in proteolytic processing and activation of Ci (*Drosophila melanogaster*) or GLI (vertebrates) transcription factors. These core pathway components are conserved between *Drosophila* and vertebrates. However, there are several key differences, such as the requirement for the primary cilium in vertebrate signaling. Importantly, the mechanisms that translate HH morphogen gradients into cell fate decisions are far from established.

Genetic studies have demonstrated that GPCR kinase 2 (GRK2) is required for HH signaling in *Drosophila* and zebrafish *(1, 2)* yet surprisingly the *Drosophila* homolog, Gprk2, was shown to promote the internalization and degradation of Smo *(3–5)*. These apparently contradictory roles, although not uncommon in the HH pathway, have posed a challenge in dissecting GRK2 function. Li *et al*. directly addressed the latter observation and propose that Gprk2 promotes Smo internalization by E3 ligase-mediated ubiquitination in *Drosophila(6)*. They identified the Smurf family of E3 ligases as required for Smo ubiquitination through an RNA interference (RNAi)-based screen and demonstrated that Smo accumulates in Smurf knockdown cells, consistent with ubiquitination promoting its turnover.

Through overexpression and knockdown experiments, Li *et al*. found that Smurf also controls Smo abundance in *Drosophila* wing disc cells. Moreover, co-immunoprecipitation experiments revealed that addition of HH ligand abolishes the association of Smurf and Smo by stimulating phosphorylation of the Smo C-terminal tail. Using truncation constructs, Li *et al*. demonstrated that the Smurf HECT domain is responsible for its recruitment to Smo. Because Gprk2 was previously shown to reduce Smo membrane accumulation, Li *et al.* tested its involvement in Smo ubiquitination and found that it cooperated with Smurf. To

explore this interaction further, Li *et al.* used co-immunoprecipitation to demonstrate that Gprk2 knockdown reduces the amount of full-length Smurf associated with Smo; this was not observed for a construct expressing the HECT domain only, indicating a potential autoinhibitory role for the N-terminus of Smurf that is regulated by Gprk2 activity. Consistently, HH ligand inhibited Gprk2-mediated Smurf phosphorylation. The authors propose that Smo functions as a scaffold for Gprk2 and Smurf that can be blocked by HH-induced Smo phosphorylation (Figure 1A). Previously, Smurf was implicated in Ptc endocytosis *(7)*; however, Li *et al.* found that Smurf-mediated Ptc ubiquitination did not depend on Gprk2. In contrast to previous findings, the authors showed that knockdown of Smo increases the association of Ptc and Smurf, suggesting that Smo and Ptc compete for the E3 ligase. In sum, Li *et al.* reveal that in the absence of HH ligand Gprk2 kinase activity promotes Smo ubiquitination and turnover by relieving autoinhibition of Smurf (Figure 1A). These findings are consistent with several reports linking Gprk2 to Smo internalization. Although genetic evidence suggests that Gprk2/GRK2 is a positive regulator of HH signaling, it appears that its role is more complex, providing another example of a HH pathway component serving apparently opposing roles. It will be important to determine whether these findings are relevant to vertebrate HH signaling, especially considering the divergence of the Smo C-terminal tail and the differential necessity of the primary cilium between species. Shedding further light on the role of GRK2, Pusapati *et al.* used mammalian model systems to investigate signaling downstream of SMO *(8)*.

An earlier study suggested that GRK2 is required to promote the ciliary accumulation of SMO, a critical step in vertebrate HH pathway activation *(4)*. However, Pusapati *et al.* and another recent study demonstrate that SMO localizes normally in GRK2-deficient fibroblasts and zebrafish embryos (2, 8). GRK2 had also been proposed to function in the internalization of the GPCR GPR161 to promote HH signaling. GPR161 can negatively regulate HH signaling during mouse neural tube development, and its ciliary localization is regulated by HH, leading to the hypothesis that GPR161 is a necessary component of the pathway. To clarify the situation Pusapati *et al.* set out to dissect signaling downstream of SMO with a focus on the epistatic relationship between GPR161, GRK2, and the G protein $G\alpha_S$. In agreement with previous genetic studies, the authors show that *Gpr161* knockout fibroblasts have heightened sensitivity to HH ligand, although basal signaling was unaffected. Consistently, GPR161 overexpression blocked HH signaling. These effects depended on the activity of SMO and GRK2, suggesting that GPR161 loss alone is not sufficient to activate the pathway through the cyclic AMP (cAMP) sensor protein kinase A (PKA) (Figure 1B). In contrast, loss of GPR161 did affect baseline pathway activity in neural progenitor cells (NPCs), probably through loss of the transcriptional repressor GLI3R, a proteolytically processed form of GLI3. In high-level HH signaling conditions, where activating GLI2A predominates, the influence of GPR161 deletion was again dependent upon SMO and GRK2, indicating that GPR161 affects the pathway in a context-dependent manner (Figure 1B). Therefore the authors suggest GPR161 functions as an attenuator rather than an essential component of HH signaling (Figure 1B), a proposal consistent with the less severe phenotype of *Gpr161* knockout mice than that of core negative regulators such as *Sufu*.

If GRK2 does not obligately function through GPR161 or affect Smo ciliary localization, then how does it regulate HH signaling? By activating the HH pathway at key nodes the authors were able to map the epistatic relationship of GRK2 to other components of the pathway using both genetic and pharmacological intervention. GRK2 deletion or inhibition interfered with SMO-stimulated pathway activity, but had no effect on pathway activation by deletion of the downstream components $G\alpha_S$ or SUFU. Studies in NPCs revealed a similar story, placing GRK2 downstream of SMO, but upstream of both $G\alpha_S$ and SUFU (Figure 1B). Although the precise substrates of GRK2 remain to be determined in vertebrates, a reasonable explanation is that GRK2 acts downstream of SMO, at the level of $G\alpha_S$-coupled GPCRs including GPR161. Overall, the combined activity of $G\alpha_i$- and $G\alpha_S$-coupled GPCRs

regulated by GRK2 would set the sensitivity of cells to HH stimulation through cell type–specific effects on PKA activity. In combination, these two studies provide mechanistic insight into the role of GRK2 and will inspire new avenues of research. Are the effects of GRK2 downstream of SMO all indirect, or does GRK2 act directly on GPCR-like SMO as originally reported *(9)* and like in *Drosophila (5, 6)*? Given that GPR161 is regulated by HH signaling, it will be important to determine whether other GPCRs are similarly affected. Finally, there is clearly extensive crosstalk between GPCRs and HH signaling. Because GRK2 has also been implicated in WNT signaling *(10)*, this raises the intriguing possibility that GPCRs and GRK2 activity could set the tone for other developmental pathways in addition to HH.

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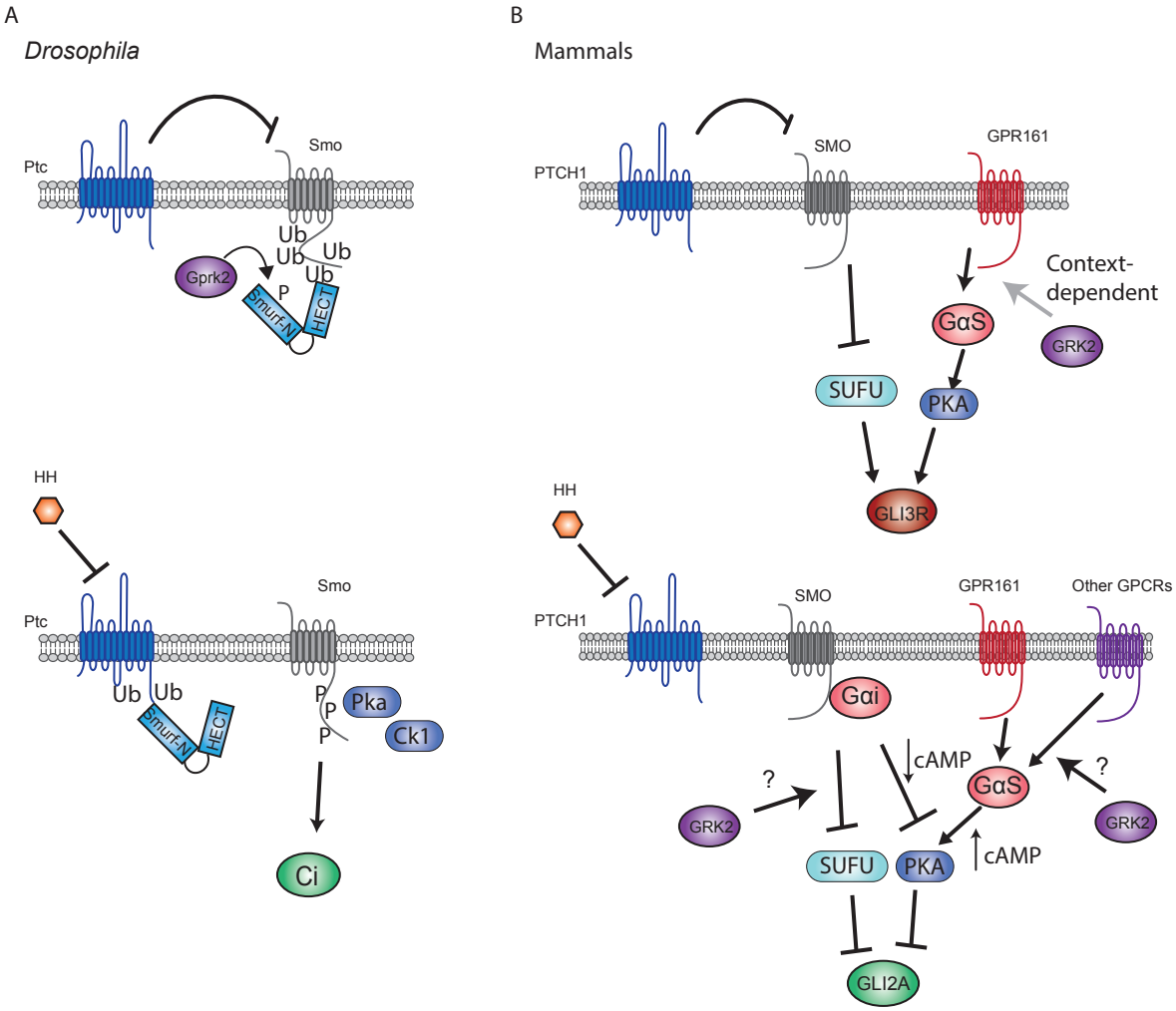

Fig. 1. The role of Gprk2/GRK2 in Hedgehog signaling.
(**A**) Experiments by Li *et al.* suggest that in the absence of HH ligand (upper panel), *Drosophila* Gprk2 phosphorylates the N-terminal domain the E3 ligase Smurf. This relieves autoinhibition of the Smurf HECT domain, allowing it to interact with Smo resulting in ubiquitination and subsequent degradation of Smo. Upon HH binding to Ptc (lower panel), phosphorylation of the Smo C-terminal tail leads to dissociation of Smurf from Smo, leaving Smurf free to interact with and ubiquitinate Ptc. Ptc binds to the WW domains in the N terminus of Smurf in a manner that does not depend on Gprk2-mediated phosphorylation of Smurf. (**B**) Work from Pusapati *et al.* indicates that under conditions of low HH signaling in vertebrates (upper panel), the role of GPR161 may be context-dependent, related to the balance of the GLI3R (repressive) and GLI2A (activating) transcription factors. In the context of high HH signaling (lower panel), GPR161 loss increases the sensitivity of cells to HH ligand in a manner that depends on SMO and GRK2, leading to the proposal that GPR161 functions as a signal attenuator. GRK2 appears to function between SMO and SUFU, as well as upstream of $G\alpha_S$, suggesting that signaling through multiple GPCRs, in addition to GPR161, could set the sensitivity of cells to activation by HH ligand.