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Hedgehog reciprocally controls trafficking of Smo and Ptc through the Smurf family of E3 ubiquitin ligases

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Abstract

Hedgehog (Hh) induces signaling by promoting the reciprocal trafficking of its receptor Patched (Ptc) and the signal transducer Smoothened (Smo), which is inhibited by Ptc, at the cell surface. We identified Smurf family E3 ubiquitin ligases as essential for Smo ubiquitination and cell surface clearance and demonstrated that Smurf family members mediate the reciprocal trafficking of Ptc and Smo in *Drosophila melanogaster*. G-protein-coupled-receptor-kinase 2 (Gprk2)-mediated phosphorylation of Smurf promoted Smo ubiquitination by increasing the recruitment of Smurf to Smo, whereas protein kinase A (PKA)-mediated phosphorylation of Smo caused Smurf to dissociate from Smo, thereby inhibiting Smo ubiquitination. Smo and Ptc competed for the same pool of Smurf family of E3 ligases, and Hh promoted Ptc ubiquitination and degradation by disrupting the association of Smurf family E3s with Smo and stimulating their binding to Ptc. Our study identifies the E3 ligases that target Smo and provides insight into how Hh regulates the reciprocal trafficking of its receptor and signal transducer.

One-Sentence Summary:

The ubiquitin E3 ligase Smurf controls the reciprocal trafficking of the Hh signaling mediators Smoothened and Patched.

Editor's Summary:

Smurf controls reciprocal trafficking of Smo and Ptc

Hedgehog (Hh) stimulates intracellular signaling by binding to Patched (Ptc) on the cell surface, thus relieving Ptc-mediated repression of the transmembrane protein and signal transducer Smoothened (Smo). In the absence of Hh, Smo is both inactivated and targeted for ubiquitination and removal from the membrane. In the presence of Hh, Ptc is targeted for ubiquitination and degradation. Li *et al.* identified the Smurf family of ubiquitin E3 ligases as required for this reciprocal regulation of Ptc and Smo in *Drosophila melanogaster*. The ability of Smurf to bind to

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and ubiquitinate Smo depended on phosphorylation of Smurf by G-protein-coupled-receptor-kinase 2 (Gprk2) and was inhibited by Hh-induced phosphorylation of Smo by protein kinase A (PKA). The Hh-induced dissociation of Smurf from Smo freed Smurf to interact with Ptc, thus promoting ubiquitination and degradation of Ptc. These findings identify Smurf family E3 ligases as key players in the reciprocal accumulation of Smo and Ptc at the cell surface.

Introduction

Hedgehog (Hh) signaling plays critical roles in embryonic development and adult tissue homeostasis in species ranging from insects to mammals (1–3). Aberrant Hh pathway activity contributes to a wide range of human disorders including birth defects and cancer (1, 4, 5). Hh controls cell growth and differentiation through a conserved signal transduction pathway that culminates in the activation of latent transcription factors Cubitus interruptus (Ci) and Gli proteins (1, 2, 6, 7). The core reception system for Hh signaling comprises two multi-span transmembrane proteins: the twelve-transmembrane protein Patched (Ptc) and the seven-transmembrane protein Smoothened (Smo), which is a member of the G protein–coupled receptor (GPCR) family. Ptc inhibits Smo in the absence of Hh. Hh binds to Ptc to release its inhibition of Smo, leading to Smo phosphorylation and activation in the presence of Hh (8).

The activity of Smo is controlled through changes in its conformation and subcellular localization. In vertebrates, cilium-localized Ptc restricts Smo ciliary accumulation and keeps Smo in an inactive conformation, whereas Hh binding to Ptc triggers its ciliary exit, leading to ciliary accumulation of phosphorylated Smo that adopts an active conformation (9–11). In *Drosophila melanogaster*, most cells lack primary cilia, but Ptc inhibits Smo phosphorylation and cell surface accumulation, whereas Hh promotes Smo phosphorylation, cell surface accumulation, and active conformation (12–14). In addition, Hh binding to Ptc facilitates internalization of the Hh-Ptc protein complex, a process required for restricting the range of Hh signaling (12, 15–18). Although the Smurf family of E3 ubiquitin ligases has been implicated in the regulation of Ptc ubiquitination and trafficking in both *Drosophila* and mammals (19–22), the mechanism underlying the regulation of Smo trafficking and cell surface accumulation is still poorly understood. In addition, how Hh signaling coordinates the reciprocal trafficking of Ptc and Smo remains unknown.

Previous studies revealed that the ubiquitination and subsequent degradation of Smo through both proteasome- and lysosome-dependent mechanisms are responsible for preventing its cell surface accumulation in the absence of Hh (23–25). Upon stimulation, Hh induces phosphorylation of the intracellular C-terminal tail of Smo (SmoCT) by protein kinase A (PKA) and casein kinase 1 (CK1), which inhibits Smo ubiquitination, thereby promoting its cell surface accumulation (23, 24). Furthermore, Hh induces sumoylation of SmoCT at Lys⁸⁵¹, which facilitates the recruitment of the deubiquitinase USP8 to antagonize Smo ubiquitination independently of PKA- and CK1-mediated phosphorylation (26). In addition to ubiquitination, the Smo-interacting proteins Kurtz (Krz), the *Drosophila* homolog of β -arrestin 2, and G protein–coupled receptor kinase 2 (Gprk2), the *Drosophila* homolog of GRK2, promote Smo internalization through unknown mechanisms (23, 27–30).

How phosphorylation of Smo inhibits its ubiquitination has remained a mystery. It has been speculated that phosphorylation of Smo may preclude the binding of an E3 ubiquitin ligase(s) (23); however, previous genetic and RNA interference (RNAi) screens have not identified any E3 ubiquitin ligase that regulates Smo activity or trafficking (31–34). One possibility is that multiple E3 ligases are involved in the regulation of Smo ubiquitination so that perturbation of individual E3s may not result in an obvious change in Smo abundance and Hh pathway activity. Furthermore, Smo ubiquitination could be catalyzed by E3 ligases that are not dedicated for Smo such that their inactivation might cause pleiotropic phenotypes. Therefore, we decided to carry out an in vitro RNAi screen using a cell-based Smo ubiquitination assay (23). From this screen, we identified the Smurf family of HECT domain-containing E3s as Smo ubiquitin ligases. We found that Smurf bound to the Smo autoinhibitory domain (SAID) through its HECT domain to promote Smo ubiquitination. Hh-induced and PKA-mediated phosphorylation of SAID dissociated Smurf from Smo, thereby inhibiting Smo ubiquitination. We found that the N-terminal region of Smurf bound to its C-terminally localized HECT domain to prevent Smurf from binding to Smo. Gprk2-mediated phosphorylation of the N-terminal region of Smurf alleviated this autoinhibition and freed the HECT domain for binding to Smo. Smo and Ptc competed for the same pool of Smurf family E3s, and Hh promoted Ptc ubiquitination by releasing Smurf family members from Smo and further stimulating their binding to Ptc.

Results

Cell-based RNAi screen identifies Smurf family members as Smo ubiquitin ligases

To identify E3 ligase(s) that promote Smo ubiquitination, we carried out an RNAi screen using a cell-based ubiquitination assay (23). We first generated a stable *Drosophila* S2 cell line expressing an inducible Myc-tagged Smo transgene under the control of the metallothionein promoter (*pMT-Myc-Smo*). Myc-Smo-expressing cells were treated with double-stranded RNAs (dsRNAs) targeting *Drosophila* E3 ligases, and cell lysates were subjected to ubiquitination assay as previously described (23, 35). We initially focused on the HECT family of E3 ubiquitin ligases because E3s in this family have been implicated in the regulation of GPCR endocytosis (36). We targeted 11 HECT domain E3s from *Drosophila* including Smurf, Nedd4, and Suppressor of deltex [Su(dx)], which together constitute the Smurf subfamily (Fig. 1A-B). Among the HECT domain E3s tested, we found that knockdown of Smurf (CG4943) reduced Smo ubiquitination (Fig. 1C). Although knockdown of either Nedd4 (CG7555) or Su(dx) (CG4244) alone did not noticeably change Smo ubiquitination, knocking them down in combination with Smurf further diminished Smo ubiquitination compared to Smurf knockdown alone (Fig. 1C-D), suggesting that the Smurf subfamily of E3s act in a partially redundant fashion to promote Smo ubiquitination. Indeed, overexpression of Smurf, Nedd4, or Su(dx), but not two other HECT-domain E3s (CG3356 and CG6190), increased Smo ubiquitination (Fig. 1E). Furthermore, mutating a critical residue in the Smurf catalytic domain (C1029A) (37) abolished its ability to promote Smo ubiquitination (Fig. 1F).

Smurf family E3 ligases reduce Smo cell surface abundance in cultured cells

Consistent with the effect on Smo ubiquitination, transfection of S2 cells with dsRNA targeting Smurf but not of dsRNAs targeting Nedd4 or Su(dx), increased cell surface abundance of Myc-Smo, and combined knockdown of Nedd4 and Su(dx) with Smurf further increased cell surface accumulation of Myc-Smo (Fig. 2A-B). Conversely, overexpression of Smurf, Nedd4, or Su(dx) decreased the cell surface accumulation as well as the overall protein abundance of a phosphomimetic form of Smo (Myc-Smo^{SD}) that contains Ser to Asp substitutions at three PKA and CK1 phosphorylation clusters (Fig. 2C-F) (13).

To examine how the Smurf family E3 ligases regulates Hh signaling responses downstream of Smo, we turned to cl-8 cells, a *Drosophila* cell line that contains all the Hh pathway components and can recapitulate Hh signaling responses as indicated by the stabilization of Smo and increased expression of *ptc*, a universal readout of Hh pathway activation (33). Consistent with the results obtained using S2 cells, knockdown of Smurf but not Nedd4 and Su(dx) increased the abundance of endogenous Smo, whereas combined knockdown of all three Smurf family members further increased Smo abundance without altering *smo* mRNA expression (Fig. 2G-I). Despite the accumulation of Smo in Smurf family knockdown cells, the expression of endogenous *ptc* mRNA and of a *ptc* luciferase (*ptc-luc*) reporter that reflects Hh pathway activation (33) was not increased (Fig. 2J-K). By contrast, cl-8 treated with Hh-conditioned medium not only accumulated Smo but also exhibited increased expression of endogenous *ptc* and the *ptc-luc* reporter gene (Fig. 2G, H, J, K). It has been shown that Hh activates signaling events downstream of Smo not only by increasing Smo cell surface abundance but also by inducing Smo to adopt an active conformation (14). The lack of pathway activation downstream of Smo in Smurf family knockdown cells implies that Smo accumulated in these cells most likely adopted an inactive conformation.

We noticed that knockdown of Smurf family members had a less dramatic effect on Smo accumulation compared to Hh stimulation (Fig. 2G, H). Therefore, it is possible that Smurf family members are not the only E3 ligases that target Smo. Indeed, we found that knockdown of Cul4, a member of the Cullin family of E3 ubiquitin ligases (38), enhanced the effect of Smurf knockdown on Smo ubiquitination and cell surface accumulation (fig. S1A-B), implying that Cul4 may act in parallel with the Smurf family to control Smo cell surface abundance. Nevertheless, we focused our effort on the Smurf family for the remainder of this study.

Smurf reduces Smo accumulation and Hh signaling in developing wings

To assess the role of Smurf in vivo, we expressed *UAS-Smurf-RNAi* (VDRC# 24681) or *UAS-Flag(Fg)-Smurf* (37) using the wing-specific Gal4 driver *MS1096* (*MS>Smurf-RNAi* or *MS>Fg-Smurf*). To maximize Gal4 activity and *UAS-RNAi* expression, larvae were grown at 29°C (39). In addition, we examined wing discs only from male larvae because *MS1096* is located on the X chromosome and males express Gal4 more abundantly than do females. We coexpressed *UAS-Dicer2* with the RNAi transgene to improve knockdown efficiency (40). Under these optimized conditions, we found that Smurf knockdown caused increased Smo accumulation in anterior compartment cells away from the anteroposterior compartment boundary (Fig. 3A-B; fig. S2A-E). Of note, the Gal4 driver *MS1096* is

expressed more strongly in the dorsal compartment cells (arrows in Fig. 3A-B) than in ventral compartment cells (arrowheads in Fig. 3A-B) (41). As a consequence, only anterodorsal cells in *MS>Smurf-RNAi* wing discs ectopically accumulated Smo due to more complete knockdown of Smurf in these cells (arrows in Fig. 3B). Similarly, *MS>Smurf-RNAi* wing discs exhibited increased Ptc abundance in anterodorsal cells near the anteroposterior boundary (Fig. 3C-D). Consistent with the experiments in cl-8 cells, knockdown of Smurf in wing discs did not induce ectopic expression of a Hh-responsive reporter gene *ptc-lacZ* (fig. S2H-I), suggesting that Smo accumulated in an inactive conformation. The accumulation of Ptc protein in anterodorsal cells near the anteroposterior boundary in Smurf knockdown wing discs is most likely due to the effect of Smurf depletion on Ptc turnover (20).

Overexpression of Smurf in *MS>Fg-Smurf* wing discs blocked Hh-induced Smo accumulation and diminished the abundance of Ptc and En in anterior compartment cells near the anteroposterior boundary (Fig. 3E-H), mostly in the dorsal regions of wing discs (arrows). Of note, En expression in anterior compartment cells near the anteroposterior boundary is induced by high levels of Hh (13). Smurf overexpression also reduced En expression in posterior compartment cells (Fig. 3G), which is not under the control of Hh. This is likely due to a pleiotropic effect of excessive Smurf activity. Coexpression of *UAS-Smurf-RNAi* with *UAS-Fg-Smurf* using the *MS1096* driver effectively eliminated Fg-Smurf expression and restored Smo accumulation in Hh-responding cells in the dorsal region of the wing discs (Fig. 3I).

Consistent with the mild effect of knockdown of Nedd4 or Su(dx) on Smo ubiquitination in S2 cells, expression of *UAS-Nedd4-RNAi* or *UAS-Su(dx)-RNAi* in wing discs under the control of *MS1096* did not cause ectopic Smo accumulation (fig. S2F-G). Wing discs with combined knockdown of Smurf, Nedd4, and Su(dx) were malformed, precluding proper analysis of Smo trafficking in wing discs that lack all the Smurf family members. In contrast to overexpression of Smurf, which inhibited Hh-induced Smo accumulation and *ptc-lacZ* expression (fig. S3A-B), overexpression of *UAS-Nedd4* or *UAS-Su(dx)* with the *MS1096* driver did not block Hh-induced Smo accumulation and *ptc-lacZ* expression (fig. S3C-D). Taken together, the results from both in vitro and in vivo experiments suggest that Smurf may play a more prominent role than Nedd4 or Su(dx) in the regulation of Smo ubiquitination and cell surface expression. For this reason, we focused on Smurf but extended some of the key findings to Nedd4 and Su(dx).

Hh inhibits Smo ubiquitination by dissociating Smurf from Smo

Binding of E3 ubiquitin ligases to their substrates often serves as a regulatory step in the control of protein ubiquitination (42). Smurf contains a C2 domain in its N-terminal region, a HECT domain in its C-terminal region, and three WW domains in the middle part of the protein (Fig. 4A). As a first step to determine how Hh inhibits Smo ubiquitination, we asked whether Smurf interacted with Smo in a Hh-dependent manner using coimmunoprecipitation (CoIP) assays. When coexpressed in S2 cells, Flag-tagged Smurf (Fg-Smurf) coimmunoprecipitated with Myc-Smo, and treating cells with Hh-conditioned medium diminished the amount of Fg-Smurf that coimmunoprecipitated with Myc-Smo (Fig. 4B).

Deletion of the Smo C-tail (Myc-Smo^{CT}) abolished the association between Smo and Smurf (Fig. 4B), suggesting that Smurf binds Smo through the C-tail. Substitution of the three Ser residues in the Smo C-tail that are phosphorylated by PKA (Ser⁶⁶⁷, Ser⁶⁸⁷, and Ser⁷⁴⁰) with Ala (Myc-Smo^{SA}) inhibited Hh-induced dissociation of Smurf from Smo (Fig. 4C). On the other hand, substitution of the three PKA phosphorylation sites and the six adjacent CK1 sites with Asp to mimic phosphorylation (Myc-Smo^{SD}) inhibited the association between Smo and Smurf regardless of the presence of Hh (Fig. 4C). These results demonstrate that Hh-induced phosphorylation of SmoCT by PKA and CK1 is both necessary and sufficient to inhibit Smurf recruitment. Similarly, both Hh and PKA-mediated phosphorylation inhibited the recruitment of Su(dx) and Nedd4 to Smo (fig. S4A-B). Taken into consideration with our previous finding that ubiquitination of Myc-Smo^{SA} was resistant to Hh inhibition, whereas Myc-Smo^{SD} exhibited low basal ubiquitination (23), these results suggest that Hh inhibits Smo ubiquitination by inhibiting the recruitment of the Smurf family of E3 ligases through PKA-mediated phosphorylation of SmoCT.

To identify the Smurf domain responsible for Smo binding, we generated several truncated forms of Smurf (Fig. 4A) and used these in CoIP experiments. Deletion of the C2 domain (Smurf^{C2}), WW domains (Smurf^{WW}), or both the C2 and WW domains (Smurf-HECT) did not affect Smo binding; however, deletion of the HECT domain (Smurf^{HECT}) abolished Smo binding, suggesting that the HECT domain of Smurf is both necessary and sufficient to mediate binding to Smo (Fig. 4D). Consistent with this, coexpression of Smurf-HECT with Myc-Smo increased Smo ubiquitination (Fig. 4E).

The middle part of SmoCT (aa661–818) contains an autoinhibitory domain (SAID) that includes the three PKA and CK1 phosphorylation clusters mutated in Myc-Smo^{SA} and Myc-Smo^{SD} (14). When fused to a heterologous protein such as the Wnt receptor Fz2 to generate Fz2-SAID (FS), the SAID domain confers robust ubiquitination of FS (23), suggesting that the SAID domain might recruit ubiquitin ligases to Smo. Indeed, we found that both full-length Smurf and its HECT domain coimmunoprecipitated with Myc-FS (Fig. 4F-G), whereas deletion of the SAID domain from Smo (Smo^{SAID}) abolished Smurf recruitment (Fig. 4H). In addition, the phospho-deficient variant of FS (FS^{SA}) exhibited increased binding to Smurf-HECT, whereas the phospho-mimicking variant (FS^{SD}) exhibited decreased binding to both full-length Smurf and its HECT domain (Fig. 4F-G). Therefore, the Smo SAID domain recruits the HECT domain of Smurf, and Hh inhibits Smurf recruitment to Smo by inducing phosphorylation of SAID by PKA and CK1.

Gprk2 regulates Smo ubiquitination and cell surface expression by promoting Smurf recruitment

Gprk2 plays a dual role in Hh signaling (28, 30). In the presence of Hh, Gprk2 phosphorylates Smo and facilitates dimerization or oligomerization of SmoCT to promote high levels of Smo activity (28, 43). However, in the absence of Hh, Gprk2 reduces Smo cell surface abundance through an unknown mechanism (28, 30). We found that knockdown of Gprk2 reduced ubiquitination of Myc-Smo in S2 cells and that combined knockdown of Gprk2 and Smurf resulted in a more dramatic reduction of Myc-Smo ubiquitination than single knockdown (Fig. 5A). On the other hand, overexpression of Gprk2, but not of the

kinase-dead mutant Gprk2^{KM}, enhanced Myc-Smo ubiquitination (Fig. 5B). These results suggest that Gprk2 reduces cell surface abundance of Smo, at least in part, by promoting Smo ubiquitination in a manner that depends on Gprk2 kinase activity. This is consistent with our previous finding that Gprk2^{KM} failed to prevent Smo accumulation in *gprk2* mutant wing discs (28).

To further determine the functional relationship between Gprk2 and Smurf in the regulation of Smo cell surface accumulation, we carried out genetic interaction experiments. We used *brinker-Gal4* (*brk-Gal4*), which is expressed in the peripheral regions of wing discs (Fig. 5C) (44), to drive expression of *UAS-Gprk2-RNAi* and *UAS-Smurf-RNAi* alone and in combination. Knockdown of Smurf (*brk>Smurf-RNAi*) had little if any effect on the abundance of Smo, whereas knockdown of Gprk2 (*brk>Gprk2-RNAi*) only slightly increased Smo abundance in the anterior *brk* expression domain where there was no Hh (Fig. 5D-E compared with Fig. 5C). However, combined knockdown of Smurf and Gprk2 resulted in a greater increase in Smo in the anterior *brk* expression domain (Fig. 5F), indicating that Gprk2 and Smurf cooperatively regulate Smo cell surface abundance.

We next determined whether Gprk2 promotes Smo ubiquitination by regulating the binding of Smurf to Smo. Knockdown of Gprk2 reduced the amount of Myc-Smo that was associated with Fg-Smurf in S2 cells (Fig. 5G). Expression of Gprk2, but not Gprk2^{KM}, restored the interaction between Smurf and Smo in S2 cells in which the endogenous Gprk2 was depleted by dsRNA targeting the 5' UTR of *gprk2* (Fig. 5H), indicating that Gprk2 promotes Smurf recruitment depending on its kinase activity. The regulation of the association between Smo and Smurf by Gprk2 requires the N-terminal region of Smurf because Gprk2 RNAi did not reduce the binding of Smurf-HECT to Smo (Fig. 5I), implying that Gprk2 may promote Smurf binding to Smo by releasing an inhibitory effect imposed by the Smurf N-terminal region.

Gprk2-mediated phosphorylation of Smurf is promoted by Smo but inhibited by Hh

We hypothesized that the N-terminal region of Smurf prevents the HECT domain from binding to Smo and that Gprk2-mediated phosphorylation of Smurf relieves this autoinhibition. Indeed, coexpression of Fg-Smurf with Myc-Gprk2 but not Myc-Gprk2^{KM} in S2 cells resulted in a mobility shift of Fg-Smurf on SDS-PAGE, which was abolished by phosphatase treatment (Fig. 6A-B), suggesting that Gprk2 promotes Smurf phosphorylation. The Gprk2-induced mobility shift of Smurf was inhibited by treating cells with Hh-conditioned medium or by knockdown of Smo (Fig. 6A-C), suggesting that Smo promotes whereas Hh inhibits Smurf phosphorylation by Gprk2. By CoIP assays, we found that Myc-Gprk2 formed a complex with Fg-Smurf and that this association was inhibited by Hh stimulation, Ptc RNAi, or Smo RNAi (Fig. 6D). Because Smo interacts with both Smurf and Gprk2 (Fig. 4)(28), we propose that Smo functions as a scaffold to bring Gprk2 and Smurf into close proximity with one another in the absence of Hh. This facilitates Smurf phosphorylation by Gprk2, which increases the binding affinity of Smurf for Smo and allows Smurf to ubiquitinate Smo. Hh stimulates the phosphorylation of Smo, which causes Smurf to dissociate from Smo and Gprk2, thereby diminishing Gprk2-mediated phosphorylation and activation of Smurf.

Gprk2-mediated phosphorylation of Smurf blocks an intramolecular interaction that prevents Smurf from binding to Smo

To determine the effect of Gprk2-mediated phosphorylation on the ability of Smurf to bind Smo, we first identified the relevant phosphorylation sites in Smurf. By coexpressing Myc-Gprk2 with truncated Fg-Smurf variants in S2 cells, we found that Gprk2 induced a mobility shift of the Smurf^{HECT} but not of Smurf-HECT (Fig. 6E-F), suggesting that Gprk2-induced phosphorylation of Smurf occurred in its N-terminal region.

Inspection of the Smurf sequence identified a cluster of Ser residues (SEDDSSSEDS; Fig. 6G) that conforms to consensus sites for Gprk2, which, like other members of the GRK family of kinases, tends to phosphorylate Ser and Thr residues in an acidic environment (28, 45). We mutated the Ser residues in this cluster to either Ala (SA; Fig. 6G) or Asp (SD; Fig. 6G) in both full-length Smurf (Smurf^{SA} or Smurf^{SD}) and Smurf^{HECT} (Smurf^{HECT-SA}). We found that Gprk2 did not induce mobility shift of either Smurf^{HECT-SA} or Smurf^{SA} (Fig. 6E, 6G), indicating that Gprk2-mediated phosphorylation of Smurf occurred at this Ser cluster. Consistent with Gprk2-mediated phosphorylation of Smurf promoting its association between Smurf and Smo, Smurf^{SD} exhibited increased binding and ubiquitination activity toward Smo compared with wild-type Smurf, whereas Smurf^{SA} showed decreased binding to and ubiquitination of Smo (Fig. 6H-I). Furthermore, the binding of Smurf^{SD} or Smurf^{SA} to Smo no longer depended on Gprk2 (Fig. 6J). Taken together, these results demonstrate that Gprk2 promotes Smurf recruitment to and ubiquitination of Smo by phosphorylating the Smurf N-terminal region.

In considering the mechanism by which Gprk2-mediated phosphorylation of Smurf promotes its binding to Smo, we speculated that the N-terminal region of Smurf may interact with its HECT domain to interfere with the binding of the HECT domain to Smo and that this autoinhibition was prevented by Gprk2-mediated phosphorylation of Smurf. Indeed, we found that HA-Smurf-HECT coimmunoprecipitated with Fg-Smurf^{HECT} when cotransfected into S2 cells, and this association was diminished by cotransfection with Myc-Gprk2 (Fig. 6K). By contrast, the association between HA-Smurf-HECT and Fg-Smurf^{HECT-SA} was not inhibited by Gprk2 (Fig. 6K), suggesting that Gprk2-mediated phosphorylation inhibits an intramolecular interaction between the Smurf N terminus and its HECT domain. Furthermore, coexpression of Fg-Smurf^{HECT} interfered with the binding of HA-Smurf-HECT to Myc-Smo, but this inhibition was released by Gprk2 (Fig. 6L). By contrast, Gprk2 failed to release the inhibition of Smo binding to Smurf-HECT by Fg-Smurf^{HECT-SA} (Fig. 6L). Hence, the intramolecular interaction between the N and C-terminal regions, which may cause Smurf to adopt a closed conformation, inhibits Smurf recruitment to Smo, and Gprk2-mediated phosphorylation of Smurf alleviates this autoinhibition.

Hh promotes Ptc-Smurf complex formation and Ptc ubiquitination by releasing the Smurf family of E3s from Smo

Hh controls reciprocal trafficking of Ptc and Smo (12). In the absence of Hh, Ptc is internalized and degraded at modest levels, and binding of Hh to Ptc further stimulates Ptc internalization and degradation (12, 18, 19, 21, 46). Whereas ligand-independent Ptc

internalization modulates cell sensitivity to the Hh morphogen (18), internalization and degradation of Hh-bound Ptc plays a critical role in limiting the spread of the Hh ligand (15, 16). Previous studies suggested that the Smurf family of E3 ubiquitin ligases are responsible for Ptc ubiquitination and internalization (20, 21). Consistent with these findings, Hh stimulation increased ubiquitination of Myc-tagged Ptc (Myc-Ptc) in S2 cells, and Smurf RNAi inhibited both basal and Hh-stimulated Myc-Ptc ubiquitination (Fig. 7A). Nedd4 or Su(dx) RNAi also reduced Myc-Ptc ubiquitination, and combined RNAi of all three Smurf family members resulted in a greater reduction of Myc-Ptc ubiquitination than did RNAi of any one Smurf family member alone (fig. S5). Furthermore, Hh increased the binding of Myc-Ptc to Fg-Smurf (Fig. 7B) as well as to Flag-tagged Nedd4 and Su(dx) (fig. S6A-B), suggesting that Hh promotes Ptc internalization and degradation by recruiting Smurf family members to ubiquitinate Ptc.

Because Hh stimulates the dissociation of Smurf family members from Smo (Fig. 4B-C; fig. S4A-B), which should increase the pool of free Smurf family E3s that are available to bind to Ptc, we wondered whether the increase in Ptc-Smurf association and Ptc ubiquitination induced by Hh were, at least in part, mediated by Hh-induced dissociation of Smurf, Nedd4, and Su(dx) from Smo. Indeed, we found that Smo RNAi phenocopied Hh stimulation because it enhanced both the interaction between Myc-Ptc and Fg-Smurf, Fg-Nedd4, or Fg-Su(dx) and the ubiquitination of Myc-Ptc (Fig. 7A-B, fig. S6A-B). Furthermore, Smo knockdown did not block Hh-stimulated ubiquitination of Myc-Ptc (Fig. 7A) or Hh-induced binding of Fg-Smurf to Myc-Ptc (Fig. 7B), suggesting that Smo activity and signaling downstream of Smo are not required for the regulation of Ptc ubiquitination by Hh.

To determine the effect of Smo depletion on Ptc turnover in vivo, we took advantage of a Ptc reporter, *L>Ptc-GFP*, which expresses GFP-tagged Ptc under the control a ribosomal promoter (18). Although the expression of *L>Ptc-GFP* is ubiquitous, the amount of Ptc-GFP protein is lower in posterior compartment cells than in anterior compartment cells away from the anteroposterior boundary due to the presence of Hh in the posterior compartment cells (Fig. 7C) (18). The extremely low abundance of Ptc-GFP in anterior compartment cells near the anteroposterior boundary is due to the presence of high levels of endogenous Ptc that can oligomerize with Ptc-GFP and promote its internalization and degradation (18). Smo RNAi clones in wing discs reduced the abundance of Ptc-GFP when the clones were situated in the anterior compartment away from the anteroposterior boundary (arrows in Fig. 7D). By contrast, Smo RNAi clones did not change Ptc-GFP abundance in the posterior compartment where the Ptc-GFP level was already decreased in response to Hh (arrowheads in Fig. 7D).

These results suggest that Smo and Ptc may compete for binding to Smurf family members and that Smo depletion or Hh-induced dissociation of the Smurf family members from Smo increases their availability for binding to Ptc, leading to increased Ptc ubiquitination and turnover. In further support of this notion, overexpression of a Smo-cyan fluorescent protein fusion (Smo-CFP) or Smo^{SA}-CFP reduced the association between Fg-Smurf and Myc-Ptc (Fig. 7E) and diminished Myc-Ptc ubiquitination (Fig. 7F).

Because Smurf dissociated from Smo is not phosphorylated by Gprk2 and thus may adopt a closed conformation (Fig. 6), we asked whether the phosphorylation state of Smurf affects

the interaction between Ptc and Smurf. CoIP experiments indicated that Myc-Ptc interacted equally well with Fg-Smurf^{WT}, Fg-Smurf^{SA} and Fg-Smurf^{SD} (fig. S7), suggesting that Ptc binds Smurf regardless of whether or not Smurf is phosphorylated by Gprk2. This is in contrast to Smo, which preferentially binds Smurf that has been phosphorylated by Gprk2 (Fig. 6H).

It has been suggested that activation of Smurf by Smo mediates the effect of Hh on Ptc ubiquitination and degradation because overexpression of Smo^{SD} increases Ptc ubiquitination (20). Therefore, we were surprised to see that depletion of Smo also increased Ptc ubiquitination. Consistent with the observation that Smo^{SD} increases Ptc ubiquitination (20), overexpression of Smo^{SD}-CFP increased the amount of Fg-Smurf that coimmunoprecipitated with Myc-Ptc from S2 cell extracts (Fig. 7E) and enhanced Myc-Ptc ubiquitination (Fig. 7F). Our previous study revealed that Smo^{SD} can recruit PKAc to transphosphorylate wild-type Smo that dimerizes with it (47). Therefore, we hypothesized that Smo^{SD} may stimulate phosphorylation of endogenous Smo to release Smurf, thereby mimicking the effect of Hh stimulation. Indeed, Smo^{SD}-CFP did not stimulate the binding of Fg-Smurf to Myc-Ptc, nor did it increase Myc-Ptc ubiquitination, in S2 cells in which endogenous Smo was depleted by dsRNA targeting the 5' UTR of *smo* (Fig. 7E-F). Furthermore, the increased association between Myc-Ptc and Fg-Smurf and the ubiquitination of Myc-Ptc in Smo-depleted cells were suppressed by the expression of Smo-CFP or Smo^{SA}-CFP (Fig. 7E-F), suggesting that unphosphorylated Smo competes with Ptc for the Smurf family of E3s.

Binding of Hh to Ptc further stimulates the recruitment of Smurf to Ptc independent of Smo

We noticed that, in contrast to Smo^{SD}, Hh could further stimulate Ptc-Smurf association and Ptc ubiquitination in Smo-depleted cells (Fig. 7A-B), suggesting that Hh may promote Ptc ubiquitination through a mechanism independent of Smo. One possibility is that binding of Hh to Ptc increases its binding affinity to Smurf. To test this, we took advantage of a mutant form of Ptc, Ptc^{L2}, that no longer binds Hh but retains its ability to inhibit Smo (17, 48, 49). Unlike Myc-Ptc ubiquitination, which was markedly stimulated by Hh, Myc-Ptc^{L2} ubiquitination was only marginally induced by Hh (Fig. 7G). The residual regulation of Myc-Ptc^{L2} by Hh is likely due to endogenous wild-type Ptc that can oligomerize with Myc-Ptc^{L2} (18, 19). Indeed, knockdown of endogenous Ptc by targeting its 5' and 3' UTRs completely abolished the regulation of Myc-Ptc^{L2} ubiquitination by Hh but did not affect Hh-stimulated ubiquitination of Myc-Ptc (Fig. 7G). These results demonstrate that binding of Hh to Ptc is required to stimulate Ptc ubiquitination by Smurf.

Despite Myc-Ptc^{L2} being unable to respond to Hh, Smo RNAi increased the ubiquitination of Myc-Ptc^{L2} similarly to that of Myc-Ptc in cells with endogenous Ptc depleted (Fig. 7H). Consistent with this, the binding of Fg-Smurf to both Myc-Ptc and Myc-Ptc^{L2} was increased in Smo-depleted cells (Fig. 7I), confirming that loss of Smo increases the pool of Smurf that is available for binding to Ptc. In Smo- and Ptc-depleted cells, Hh further increased the binding of Fg-Smurf to Myc-Ptc (Fig. 7I) as well as the ubiquitination of Myc-Ptc (Fig. 7H). In contrast, Hh did not increase the binding of Smurf to and ubiquitination of

Myc-Ptc^{L2} in these cells (Fig. 7H-I). These results suggest that binding of Hh to Ptc could further increase the recruitment of Smurf to Ptc even after Smurf was released from Smo.

Discussion

It has been well documented that Hh stimulates reciprocal trafficking of Ptc and Smo and that Hh-induced phosphorylation of Smo promotes Smo cell surface accumulation; however, the underlying molecular mechanisms have remained obscure. Here we identified the Smurf family of HECT-domain-containing ubiquitin E3 ligases as essential for Smo ubiquitination and cell surface clearance. We demonstrated that Hh inhibits Smo ubiquitination by preventing the recruitment of these E3 ligases through PKA-mediated phosphorylation of Smo. In addition, we provided evidence that Smo and Ptc compete for a common pool of E3s and that Hh stimulates Ptc ubiquitination by both liberating Smurf family members from Smo and further stimulating their binding to Ptc (Fig. 8).

Regulation of Smo ubiquitination and cell surface expression by multiple E3 ubiquitin ligases, Gprk2, and PKA

It has been shown that ubiquitination of Smo controls its cell surface abundance and that the Hh-induced phosphorylation of Smo by PKA inhibits Smo ubiquitination to promote its cell surface accumulation (23, 24). Hh also stimulates sumoylation of Smo, which in turn recruits USP8 to deubiquitinate Smo in a manner that does not depend on phosphorylation of Smo by PKA (26), leaving open the question of how PKA-mediated phosphorylation of Smo inhibits its ubiquitination and degradation. Using a cell-based Smo ubiquitination assay that is sensitive to perturbation, we identified the Smurf family of HECT-domain-containing E3 ubiquitin ligases, including Smurf, Nedd4, and Su(dx), as Smo E3s. Functional studies employing both gain- and loss-of-function approaches revealed that Smurf plays a more prominent role than Nedd4 or Su(dx) in the regulation of Smo ubiquitination and cell surface accumulation.

The Smurf family E3s contain multiple WW domains that normally bind to substrates containing a PPXY-motif, as is the case for Ptc (19–21). However, we found that the region containing all three WW domains of Smurf was dispensable for its binding to Smo. Instead, Smurf interacted with Smo through its HECT domain. Indeed, a truncated form of Smo consisting of only the HECT domain (Smurf-HECT) can bind and ubiquitinate Smo. The interaction between Smurf-HECT and Smo is inhibited by the N-terminal region of Smurf, and this autoinhibition is alleviated by Gprk2-mediated phosphorylation of a Ser cluster near the N-terminal region of Smurf. Depletion of Gprk2 compromised Smurf recruitment to Smo and the subsequent ubiquitination of Smo in S2 cells, which explains, at least in part, why Smo abundance is increased in *gprk2* mutant or Gprk2 RNAi wing discs (28–30). Hence, Gprk2 regulates Smo trafficking by increasing a local pool of phosphorylated Smurf that is competent to bind to and ubiquitinate Smo (Fig. 8). We propose that Smurf may adopt two different conformations depending on its phosphorylation state. Unphosphorylated Smurf adopts a closed conformation in which the N-terminal region interacts with the C-terminal region to prevent its interaction with Smo. Gprk2-mediated phosphorylation of the N-terminal region disrupts its interaction with the C-terminal region, allowing Smurf to

adopt an open conformation capable of binding to Smo. In contrast, Gprk2-mediated phosphorylation of Smurf did not seem to affect its interaction with Ptc, because Ptc bound to wild-type, phosphorylation-deficient, and phosphomimetic forms of Smurf equally well (fig. S7). Because Ptc interacts with the WW domains of Smurf (20), this observation suggests that the WW domains of Smurf are likely to be exposed in both the closed and open conformations. An analogous conformational change has been attributed to the regulation of the E3 ubiquitin ligase Itch by Jun N-terminal kinase 1 (JNK1), in which phosphorylation of Itch disrupts an inhibitory interaction between its WW and HECT domains (50). However, in the case of Itch, the phosphorylation-induced conformational change appears to enhance its catalytic activity rather than increase substrate binding. It would be interesting to determine whether other HECT-domain-containing E3s also recognize substrates through their HECT domains in a manner that is regulated by autoinhibition and phosphorylation.

Smo recruits Smurf through the Smo autoinhibitory domain (SAID) located in the Smo C-tail, which explains, at least in part, why deletion of SAID resulted in Smo cell surface accumulation, whereas addition of SAID to Fz2 resulted in ubiquitination and internalization of the fusion protein (14, 23). SAID contains three Ser clusters phosphorylated by PKA and CK1 upon Hh stimulation (14). Phosphomimetic mutations of the three PKA and CK1 phosphorylation clusters (Smo^{SD}) diminished the binding of Smurf, Su(dx), and Nedd4 to Smo whereas phospho-deficient mutations of the three PKA sites (Smo^{SA}) prevented Hh-induced Smurf dissociation from the mutant Smo. In conjunction with our previous findings that Smo^{SD} exhibited diminished ubiquitination and accumulated at high levels on the cell surface in the absence of Hh, whereas Smo^{SA} was ubiquitinated and failed to accumulate on the cell surface even after Hh stimulation (13, 23), these results suggest that Hh-stimulated phosphorylation of Smo by PKA and CK1 inhibits Smo ubiquitination by dissociating the Smurf family of E3s from Smo.

The phenotypes associated with Smurf knockdown were weak in general. Indeed, in order to observe ectopic Smo accumulation in wing discs, we had to optimize the RNAi efficiency (Fig. 3), which may explain why a previous study failed to observe this phenotype (20). Aside from the partially redundant functions of Nedd4 and Su(dx), additional E3(s) may also participate in the regulation of Smo cell surface accumulation, which could explain why the effect of combined knockdown of all the Smurf family members on Smo accumulation was less dramatic compared with Hh stimulation (Fig. 2E). Indeed, knockdown of Cul4 enhanced the effect of Smurf depletion on Smo ubiquitination and cell surface expression (fig. S1), suggesting that Cul4 may act in parallel with the Smurf family to regulate Smo ubiquitination and trafficking. Further study is needed to determine how Cul4 regulates Smo ubiquitination and how this regulation is inhibited by Hh.

It has been shown that Hh stimulates sumoylation of Smo in a manner that does not depend on Smo phosphorylation by PKA and that sumoylation recruits USP8 to deubiquitinate Smo (26, 51). Hence, Hh regulates Smo trafficking and cell surface expression through two paralleled mechanisms: PKA-dependent inhibition of ubiquitination and PKA-independent but sumoylation-dependent deubiquitination. We speculate that the involvement of multiple parallel regulatory mechanisms that control Smo trafficking and cell surface expression may allow graded Hh signals to fine-tune the subcellular localization and abundance of Smo to

generate precise signaling outputs. It would be interesting to determine whether these two mechanisms are differentially regulated by graded Hh signals.

The Smurf family of E3s mediates the reciprocal trafficking of Smo and Ptc

It is interesting that the Smurf family of E3 ligases also regulates the trafficking of Ptc (19–21). Like the regulation of Smo, Smurf also appears to play a more prominent role in the regulation of Ptc ubiquitination and turnover than the other Smurf family members (fig. S5) (20), raising an important question of how Hh signaling coordinately regulates the activity of the Smurf family members to achieve appropriate regulation of reciprocal trafficking of Ptc and Smo. Here we demonstrate that Hh inhibits the ubiquitination of Smo while enhancing the ubiquitination of Ptc by switching Smurf substrates from Smo to Ptc. Mechanistically, we found that Smo and Ptc compete for the same pool of Smurf and that Gprk2-mediated phosphorylation of Smurf increases its binding to Smo, thus limiting the amount of Smurf that is accessible to Ptc. Binding of Hh to Ptc alleviates its inhibition of Smo, allowing PKA to phosphorylate Smo to release Smurf, thus increasing the amount of free Smurf accessible to Ptc (Fig. 8). Indeed, depletion of Smo increased the amount of Smurf bound to Ptc, and consequently the ubiquitination of Ptc in S2 cells, and decreased Ptc abundance in wing discs, which mimics the effect of Hh stimulation. In addition, Hh further increased Ptc-Smurf association and Ptc ubiquitination in Smo-depleted cells, and this stimulation depended on the binding of Ptc to Hh, suggesting that Hh-bound Ptc may have increased binding affinity for Smurf. It is possible that binding of Hh to Ptc changes the conformation of Ptc or elicits a modification of its intracellular tail to facilitate Smurf binding. Because both Hh stimulation and Smo deletion increased the association of Ptc with Su(dx) and Nedd4 (fig. S6), it is likely that the mechanism underlying Smurf regulation could also be extended to Su(dx) and Nedd4.

A previous study suggested that Smo activates Smurf to regulate Ptc turnover in response to Hh because an constitutively active form of Smo (Smo^{SD}) promotes Ptc ubiquitination (20). However, we showed that depletion of Smo did not block Hh-stimulated ubiquitination of Ptc, nor did it inhibit the binding of Smurf to Ptc (Fig. 7A-B), suggesting that Smo activity per se is not required for Hh-stimulated ubiquitination of Ptc. Because our previous study demonstrated that Smo can form dimers or oligomers and Smo^{SD} can recruit PKAc to transphosphorylate the wild-type Smo that dimerizes with it (47), it is conceivable that overexpression of Smo^{SD} induces phosphorylation of endogenous Smo by PKA to release Smurf, thereby increasing the accessibility of Smurf to Ptc. In support of this view, depletion of Smo phenocopied overexpression of Smo^{SD} in the regulation of Ptc-Smurf complex formation and Ptc ubiquitination. Furthermore, overexpression of Smo^{SD} had no effect on Ptc-Smurf interaction and Ptc ubiquitination in Smo-depleted cells (Fig. 7E-F). Hence, Smo^{SD} acts through a de-repression mechanism, alleviating the inhibition of Ptc ubiquitination by endogenous Smo. Because other Smurf family members including Su(dx) and Nedd4 regulate both Ptc and Smo ubiquitination and their interactions with Ptc and Smo are reciprocally regulated by Hh, we propose that Hh signaling may redirect multiple E3s from Smo to Ptc to regulate their reciprocal trafficking. It is interesting to note that Hh also redirects PKA and CK1 to reciprocally regulate the phosphorylation of Smo and Ci (47, 52). Hence, employment of the same set of enzymes to reciprocally regulate the activities of

different pathway components in signaling “on” and “off” states is a recurring theme in Hh signal transduction.

Vertebrate Hh signaling also affects reciprocal trafficking of Ptc and Smo, with Ptc exiting from the primary cilium while Smo moves in (9, 10). It has been shown that the mammalian Smurf family members Smurf1 and Smurf2 promote Ptc1 ubiquitination and that Smurf-mediated endocytosis of Ptc1 is required for its ciliary clearance and proper Hh signaling (22). Although it is not clear whether the ubiquitination pathway is involved in the regulation of mammalian Smo ciliary trafficking, it has been shown that sumoylation of mammalian Smo facilitates its ciliary localization (26). Therefore, it would be interesting to determine in the future whether Smo trafficking is regulated by ubiquitination and whether Hh regulates the reciprocal trafficking of Smo and Ptc1 in mammals through common E3 ligases.

Materials and Methods

Constructs and transgenes

The following transgenic fly stocks were used: *UAS-Smurf-RNAi* (VDRC#24681), *UAS-Su(dx)-RNAi* (VDRC#21814; VDRC#103814), *UAS-Nedd4-RNAi* (VDRC#13121; BL#34741), *UAS-Gprk2-RNAi* (BL#34843), *UAS-Smurf-Flag* (37), *UAS-Nedd4* and *UAS-Su(dx)* (53), *L>Ptc-GFP* (18), *ptc-lacZ* (2nd and 3rd chromosomal insertion lines) (13), *MS1096-Gal4* (54), and *brk-Gal4* (BL#53707). The *UAS-Smo* constructs Myc-Smo, Myc-Smo^{SA} (Smo^{SA123}), Myc-Smo^{SD} (Smo^{SD123}), Myc-Smo^{CT}, Myc-Smo^{SAID}, Smo-CFP, Smo^{SA}-CFP, Smo^{SD}-CFP were previously described (13, 14, 55). The Fz2 and Fz2-Smo fusion constructs Myc-Fz, Myc-FS, Myc-FS^{SA}, and Myc-FS^{SD} were previously described (23). Fg-Gprk2, Fg-Gprk2^{KM}, Myc-Gprk2, and Myc-Gprk2^{KM} were previously described (28). DNA constructs for Fg-Smurf and its deletion mutants WW, HECT, C2, WW, HECT, Fg-Nedd4, and Fg-Su(dx) were generated by subcloning the corresponding cDNA coding sequence into the *pUAST* vector containing a Flag tag (56). To construct HA-Smurf-HECT, the corresponding cDNA fragment was amplified by PCR and subcloned in frame with three copies of HA tags in the *pUAST-3HA* vector (56). PCR-based site directed mutagenesis was used to generate Smurf^{SA}, Smurf^{SD}, and Smurf^{HECT-SA}. Myc-Ptc has six copies of Myc tags fused to the C-terminus of full-length Ptc. For making *UAS-Myc-Ptc^{L2}*, Ptc^{L2} coding sequence was amplified from Ptc^{L2}-GFP (kindly provided by Andrea Casali) to replace Ptc coding sequence in *UAS-Myc-Ptc*.

Cell culture and transfection

Drosophila S2 cells were cultured in *Drosophila* SFM (Invitrogen) with 10% fetal bovine serum, 100 U/ml of penicillin, and 100mg/ml of streptomycin at 23°C. cl-8 cells were cultured in Shields and Sang M3 Insect Medium (Sigma) with 2.5% fetal bovine serum, 2.5% fly extract, insulin (0.125 IU/ml; 0.5 mg/ml) (Sigma), penicillin (100 U/ml), and streptomycin (100 mg/ml) at 24°C. Transfection was carried out using the Calcium Phosphate Transfection Kit (Specialty Media) according to manufacturer's instructions. HhN-conditioned medium treatment was carried out as described (33). Briefly, S2 cells stably expressing the N terminal fragment of Hh (HhN) that is active for signaling were

selected in 200 µg/ml hygromycin. Hh-conditioned medium was prepared by culturing cells without hygromycin but with 0.7 mM CuSO₄ for one day. The medium was harvested and sterilized by filtration. Unless mentioned otherwise, Hh-conditioned medium was used at a 6:4 dilution ratio by fresh medium.

Immunoprecipitation, Western blotting, and immunostaining

Immunoprecipitation and Western blot analysis were carried out using standard protocols as previously described (57). Cells were treated with 50µM MG132 (Calbiochem) for 4h to stabilize or 20mM NH₄Cl (sigma) for 18h to stabilize Ptc prior to harvesting and lysing the cells. For Smo cell surface staining assay, S2 cells stably expressing Myc-Smo were harvested and washed with PBS, fixed with 4% formaldehyde at room temperature for 20 min, and incubated with the mouse anti-Myc antibody in PBS at room temperature for 30 min. Cells were washed 3 times by PBS followed by secondary antibody staining. Immunostaining of imaginal discs was carried out as described (31). Antibodies used in this study were: mouse anti-EN (DSHB), mouse anti-Ptc and mouse anti-SmoN (DSHB), rabbit and mouse anti-Flag (Sigma), mouse anti-Myc (Santa Cruz), mouse anti-HA (Santa Cruz), mouse anti-Ci 2A1 (58), mouse anti-GFP (Millipore), and rabbit anti-GFP (Santa Cruz).

Quantitative RT-PCR

Total RNA was extracted from 1X10⁶ cells using RNeasy Plus mini kit (QIAGEN), and cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories). RT-qPCR was performed using iQ SYBR Green System (Bio-Rad Laboratories). RT-qPCR was performed in triplicate on each of three independent biological replicates. Primer sequences used are: 5'- ATGCAGTACTTAACTTTCCG -3' and 5'- GTAGCAACGGGCACGTCGGAC -3' (for Smo), 5'- ATGGACCGCGACAGCCTCCCA -3' and 5'- CGACGCAGAAGGTGCTCAGCA -3' (for ptc). Actin was used as a normalization control. Quantification of mRNA levels was calculated using the comparative C_T method.

RNAi and dual luciferase assay

Double-stranded (ds) RNA was generated using the MEGAscript High Yield Transcription Kit (Ambion: #AM1334) according to the manufacturer's instruction. The following primers were used for generating the dsRNA targeting each gene:

Smurf: 5'-

GAATTAATACGACTCACTATAGGGAGAAGCGGAGGAGGAAGTAGATCC-3' and 5'- GAATTAATACGACTCACTATAGGGAGATCGAATCATTGCAAAATACT-3'.

Nedd4: 5'-GAATTAATACGACTCACTATAGGGAGAGTCGCACATTTGTTGCTTCAG-3' and 5'-GAATTAATACGACTCACTATAGGGAGACCGCTTATAAAAATCATTA-3'.

Su(dx): 5'- GAATTAATACGACTCACTATAGGGAGAGGGACTGGATTATGGTGGTG and 5'- GAATTAATACGACTCACTATAGGGAGAACGCTAAACCAGAGCTCCAA-3'.

Cul4: 5'-GAATTAATACGACTCACTATAGGGAGACGCGAATTCGCTGCAAAATTC-3' and 5'-GAATTAATACGACTCACTATAGGGAGAGCGTCTAGAGCTCGTTCTCCT-3',

Smo 5' UTR:

5'-GAATTAATACGACTCACTATAGGGAGAGTCGCACATTTGTTGCTTCAG-3'

and 5'-GAATTAATACGACTCACTATAGGGAGACCGCTTATAAAAATCATTAAA-3'.

Gprk2: 5'- GAATTAATACGACTCACTATAGGGAGAGGGGGCGACGCCTTGGACGCC-

3' and 5'-GAATTAATACGACTCACTATAGGGAGAAAAATACATAGAGCTCTCAA-3',

Gprk2 5' UTR:

5'-GAATTAATACGACTCACTATAGGGAGATTTCACTAACTCAACGACGGT-3' and

5'-GAATTAATACGACTCACTATAGGGAGAAAGAACAAGAAGAAGCAGCGA-3'

Ptc 5' UTR:

5'-GAATTAATACGACTCACTATAGGGAGACAGACTGCGTGCGATCCTCGA-3' and
5'-

GAATTAATACGACTCACTATAGGGAGAGTTATTGGAATCTCGTATTTT-3',

Ptc 3' UTR:

5'-GAATTAATACGACTCACTATAGGGAGACACTAGCACTAGTTCCTGTAG-3' and 5'-

GAATTAATACGACTCACTATAGGGAGAGTACATATTTAACTAAATTA-3'.

dsRNA targeting the Fire Fly Luciferase coding sequence was used as a control. For knockdown experiments, S2 or cl-8 cells were cultured in serum-free SFM medium containing the indicated dsRNA at 23°C for 8 hrs. After adding fetal bovine serum to a final concentration of 10% (S2 cells) or 2.5% (cl-8 cells), dsRNA treated cells were cultured for 2 days prior to analysis or overnight before transfection with DNA constructs. The transfected cells were cultured for additional 2 days prior to analysis. Dual-luciferase activity was measured with Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, cl-8 cells were treated with corresponding dsRNA in serum-free culture medium for 8 hrs at 24°C, then fetal bovine serum was added to a final concentration of 2.5%. Cells were cultured for 24 hrs before transfected with the constructs of ptc-firefly luciferase and Pol III-renilla luciferase. For Hh treatment, two-thirds of the medium was replaced with Hh-conditioned medium, and cells were incubated for 24 hrs before harvest. Each sample was measured in triplicate using a FLUOstar OPTIMA plate reader (BMG Labtech).

Ubiquitination assay

Ubiquitination assays were carried out based on a protocol described previously (23). Briefly, cells were treated with 50μM MG132 (Calbiochem) for 4h to inhibit proteasome-mediated degradation or 20mM NH₄Cl(sigma) for 18h to inhibit lysosome-mediated degradation (Ptc only) prior to harvest. Cells were lysed in 100 μl of denaturing buffer (1%

SDS/50 mM Tris, pH 7.5/0.5 mM EDTA/1 mM DTT). After incubation for 5 min at 100°C, the lysates were diluted 10-fold with lysis buffer and then subjected to immunoprecipitation and Western blot analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References and Notes

1. Jiang J, Hui CC, Hedgehog signaling in development and cancer. *Dev Cell* 15, 801–812 (2008). [PubMed: 19081070]
2. Briscoe J, Therond PP, The mechanisms of Hedgehog signalling and its roles in development and disease. *Nat Rev Mol Cell Biol* 14, 418–431 (2013).
3. Ingham PW, McMahon AP, Hedgehog signaling in animal development: paradigms and principles. *Genes Dev* 15, 3059–3087. (2001). [PubMed: 11731473]
4. Villavicencio EH, Walterhouse DO, Iannaccone PM, The sonic hedgehog-patched-gli pathway in human development and disease. *Am J Hum Genet* 67, 1047–1054. (2000). [PubMed: 11001584]
5. Taipale J, Beachy PA, The Hedgehog and Wnt signalling pathways in cancer. *Nature* 411, 349–354 (2001). [PubMed: 11357142]
6. Wilson CW, Chuang PT, Mechanism and evolution of cytosolic Hedgehog signal transduction. *Development* 137, 2079–2094 (2010). [PubMed: 20530542]
7. Hui CC, Angers S, Gli proteins in development and disease. *Annual review of cell and developmental biology* 27, 513–537 (2011).
8. Chen Y, Jiang J, Decoding the phosphorylation code in Hedgehog signal transduction. *Cell Res* 23, 186–200 (2013). [PubMed: 23337587]
9. Corbit KC, Aanstad P, Singla V, Norman AR, Stainier DY, Reiter JF, Vertebrate Smoothed functions at the primary cilium. *Nature* 437, 1018–1021 (2005). [PubMed: 16136078]
10. Rohatgi R, Milenkovic L, Scott MP, Patched1 regulates hedgehog signaling at the primary cilium. *Science* 317, 372–376 (2007). [PubMed: 17641202]
11. Chen Y, Sasai N, Ma G, Yue T, Jia J, Briscoe J, Jiang J, Sonic Hedgehog dependent phosphorylation by CK1alpha and GRK2 is required for ciliary accumulation and activation of smoothed. *PLoS Biol* 9, e1001083 (2011). [PubMed: 21695114]
12. Denef N, Neubuser D, Perez L, Cohen SM, Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothed. *Cell* 102, 521–531. (2000). [PubMed: 10966113]
13. Jia J, Tong C, Wang B, Luo L, Jiang J, Hedgehog signalling activity of Smoothed requires phosphorylation by protein kinase A and casein kinase I. *Nature* 432, 1045–1050 (2004). [PubMed: 15616566]
14. Zhao Y, Tong C, Jiang J, Hedgehog regulates smoothed activity by inducing a conformational switch. *Nature* 450, 252–258 (2007). [PubMed: 17960137]
15. Chen Y, Struhl G, Dual roles for patched in sequestering and transducing Hedgehog. *Cell* 87, 553–563 (1996). [PubMed: 8898207]

16. Torroja C, Gorfinkiel N, Guerrero I, Patched controls the Hedgehog gradient by endocytosis in a dynamin-dependent manner, but this internalization does not play a major role in signal transduction. *Development* 131, 2395–2408 (2004). [PubMed: 15102702]
17. Casali A, Struhl G, Reading the Hedgehog morphogen gradient by measuring the ratio of bound to unbound Patched protein. *Nature* 431, 76–80 (2004). [PubMed: 15300262]
18. Casali A, Self-induced patched receptor down-regulation modulates cell sensitivity to the hedgehog morphogen gradient. *Sci Signal* 3, ra63 (2010). [PubMed: 20736483]
19. Lu X, Liu S, Kornberg TB, The C-terminal tail of the Hedgehog receptor Patched regulates both localization and turnover. *Genes Dev* 20, 2539–2551 (2006). [PubMed: 16980583]
20. Huang S, Zhang Z, Zhang C, Lv X, Zheng X, Chen Z, Sun L, Wang H, Zhu Y, Zhang J, Yang S, Lu Y, Sun Q, Tao Y, Liu F, Zhao Y, Chen D, Activation of Smurf E3 ligase promoted by smoothened regulates hedgehog signaling through targeting patched turnover. *PLoS Biol* 11, e1001721 (2013). [PubMed: 24302888]
21. Brigui A, Hofmann L, Arguelles C, Sanial M, Holmgren RA, Plessis A, Control of the dynamics and homeostasis of the Drosophila Hedgehog receptor Patched by two C2-WW-HECT-E3 Ubiquitin ligases. *Open Biol* 5, (2015).
22. Yue S, Tang LY, Tang Y, Tang Y, Shen QH, Ding J, Chen Y, Zhang Z, Yu TT, Zhang YE, Cheng SY, Requirement of Smurf-mediated endocytosis of Patched1 in sonic hedgehog signal reception. *Elife* 3, (2014).
23. Li S, Chen Y, Shi Q, Yue T, Wang B, Jiang J, Hedgehog-regulated ubiquitination controls smoothened trafficking and cell surface expression in Drosophila. *PLoS biology* 10, e1001239 (2012). [PubMed: 22253574]
24. Xia R, Jia H, Fan J, Liu Y, Jia J, USP8 promotes smoothened signaling by preventing its ubiquitination and changing its subcellular localization. *PLoS biology* 10, e1001238 (2012). [PubMed: 22253573]
25. Yang X, Mao F, Lv X, Zhang Z, Fu L, Lu Y, Wu W, Zhou Z, Zhang L, Zhao Y, Drosophila Vps36 regulates Smo trafficking in Hedgehog signaling. *J Cell Sci* 126, 4230–4238 (2013). [PubMed: 23843610]
26. Ma G, Li S, Han Y, Li S, Yue T, Wang B, Jiang J, Regulation of Smoothened Trafficking and Hedgehog Signaling by the SUMO Pathway. *Dev Cell*, (2016).
27. Molnar C, Ruiz-Gomez A, Martin M, Rojo-Berciano S, Mayor F, de Celis JF, Role of the Drosophila non-visual ss-arrestin kurtz in hedgehog signalling. *PLoS Genet* 7, e1001335 (2011). [PubMed: 21437272]
28. Chen Y, Li S, Tong C, Zhao Y, Wang B, Liu Y, Jia J, Jiang J, G protein-coupled receptor kinase 2 promotes high-level Hedgehog signaling by regulating the active state of Smo through kinase-dependent and kinase-independent mechanisms in Drosophila. *Genes Dev* 24, 2054–2067 (2010). [PubMed: 20844016]
29. Cheng S, Maier D, Neubueser D, Hipfner DR, Regulation of smoothened by Drosophila G-protein-coupled receptor kinases. *Dev Biol* 337, 99–109 (2010). [PubMed: 19850026]
30. Molnar C, Holguin H, Mayor F, Jr., Ruiz-Gomez A, de Celis JF, The G protein-coupled receptor regulatory kinase GPRK2 participates in Hedgehog signaling in Drosophila. *Proc Natl Acad Sci U S A* 104, 7963–7968 (2007). [PubMed: 17483466]
31. Jiang J, Struhl G, Protein kinase A and hedgehog signaling in Drosophila limb development. *Cell* 80, 563–572 (1995). [PubMed: 7867064]
32. Jiang J, Struhl G, Regulation of the Hedgehog and Wingless signalling pathways by the F-box/WD40-repeat protein Slimb. *Nature* 391, 493–496 (1998). [PubMed: 9461217]
33. Lum L, Yao S, Mozer B, Rovescalli A, Von Kessler D, Nirenberg M, Beachy PA, Identification of Hedgehog pathway components by RNAi in Drosophila cultured cells. *Science* 299, 2039–2045 (2003). [PubMed: 12663920]
34. Nybakken K, Vokes SA, Lin TY, McMahon AP, Perrimon N, A genome-wide RNA interference screen in Drosophila melanogaster cells for new components of the Hh signaling pathway. *Nat Genet* 37, 1323–1332 (2005). [PubMed: 16311596]

35. Zhang Q, Zhang L, Wang B, Ou CY, Chien CT, Jiang J, A hedgehog-induced BTB protein modulates hedgehog signaling by degrading Ci/Gli transcription factor. *Dev Cell* 10, 719–729 (2006). [PubMed: 16740475]
36. Shenoy SK, Xiao K, Venkataramanan V, Snyder PM, Freedman NJ, Weissman AM, Nedd4 mediates agonist-dependent ubiquitination, lysosomal targeting, and degradation of the beta2-adrenergic receptor. *J Biol Chem* 283, 22166–22176 (2008). [PubMed: 18544533]
37. Liang YY, Lin X, Liang M, Brunicardi FC, ten Dijke P, Chen Z, Choi KW, Feng XH, dSmurf selectively degrades decapentaplegic-activated MAD, and its overexpression disrupts imaginal disc development. *J Biol Chem* 278, 26307–26310 (2003). [PubMed: 12754252]
38. Angers S, Li T, Yi X, MacCoss MJ, Moon RT, Zheng N, Molecular architecture and assembly of the DDB1-CUL4A ubiquitin ligase machinery. *Nature* 443, 590–593 (2006). [PubMed: 16964240]
39. Duffy JB, GAL4 system in *Drosophila*: a fly geneticist's Swiss army knife. *Genesis* 34, 1–15 (2002). [PubMed: 12324939]
40. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, Kinsey K, Oppel S, Scheiblaue S, Couto A, Marra V, Keleman K, Dickson BJ, A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila*. *Nature* 448, 151–156 (2007). [PubMed: 17625558]
41. Wang G, Wang B, Jiang J, Protein kinase A antagonizes Hedgehog signaling by regulating both the activator and repressor forms of *Cubitus interruptus*. *Genes Dev* 13, 2828–2837 (1999). [PubMed: 10557210]
42. Herskho A, Ciechanover A, The ubiquitin system. *Annu Rev Biochem* 67, 425–479 (1998). [PubMed: 9759494]
43. Li S, Li S, Han Y, Tong C, Wang B, Chen Y, Jiang J, Regulation of Smoothed Phosphorylation and High-Level Hedgehog Signaling Activity by a Plasma Membrane Associated Kinase. *PLoS Biol* 14, e1002481 (2016). [PubMed: 27280464]
44. Minami M, Kinoshita N, Kamoshida Y, Tanimoto H, Tabata T, brinker is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. *Nature* 398, 242–246 (1999). [PubMed: 10094047]
45. Premont RT, Inglese J, Lefkowitz RJ, Protein kinases that phosphorylate activated G protein-coupled receptors. *Faseb J* 9, 175–182 (1995). [PubMed: 7781920]
46. Zhu AJ, Zheng L, Suyama K, Scott MP, Altered localization of *Drosophila* Smoothed protein activates Hedgehog signal transduction. *Genes Dev* 17, 1240–1252 (2003). [PubMed: 12730121]
47. Li S, Ma G, Wang B, Jiang J, Hedgehog induces formation of PKA-Smoothed complexes to promote Smoothed phosphorylation and pathway activation. *Sci Signal* 7, ra62 (2014). [PubMed: 24985345]
48. Briscoe J, Chen Y, Jessell TM, Struhl G, A hedgehog-insensitive form of patched provides evidence for direct long-range morphogen activity of sonic hedgehog in the neural tube. *Mol Cell* 7, 1279–1291. (2001). [PubMed: 11430830]
49. Casali A, Batlle E, Intestinal stem cells in mammals and *Drosophila*. *Cell Stem Cell* 4, 124–127 (2009). [PubMed: 19200801]
50. Gallagher E, Gao M, Liu YC, Karin M, Activation of the E3 ubiquitin ligase Itch through a phosphorylation-induced conformational change. *Proc Natl Acad Sci U S A* 103, 1717–1722 (2006). [PubMed: 16446428]
51. Zhang J, Liu Y, Jiang K, Jia J, SUMO regulates the activity of Smoothed and Costal-2 in *Drosophila* Hedgehog signaling. *Sci Rep* 7, 42749 (2017). [PubMed: 28195188]
52. Ranieri N, Therond PP, Ruel L, Switch of PKA substrates from *Cubitus interruptus* to Smoothed in the Hedgehog signalosome complex. *Nat Commun* 5, 5034 (2014). [PubMed: 25289679]
53. Wilkin MB, Carbery AM, Fostier M, Aslam H, Mazaleyrat SL, Higgs J, Myat A, Evans DA, Cornell M, Baron M, Regulation of notch endosomal sorting and signaling by *Drosophila* Nedd4 family proteins. *Curr Biol* 14, 2237–2244 (2004). [PubMed: 15620650]
54. Capdevila J, Guerrero I, Targeted expression of the signalling molecule decapentaplegic induces pattern duplications and growth alterations in *Drosophila* wings. *EMBO Journal* 13, 4459–4468 (1994). [PubMed: 7925288]

55. Jia J, Tong C, Jiang J, Smoothened transduces Hedgehog signal by physically interacting with Costal2/Fused complex through its C-terminal tail. *Genes Dev* 17, 2709–2720 (2003). [PubMed: 14597665]
56. Tong C, Jiang J, Using immunoprecipitation to study protein-protein interactions in the Hedgehog-signaling pathway. *Methods Mol Biol* 397, 215–229 (2007). [PubMed: 18025723]
57. Zhang W, Zhao Y, Tong C, Wang G, Wang B, Jia J, Jiang J, Hedgehog-regulated costal2-kinase complexes control phosphorylation and proteolytic processing of cubitus interruptus. *Dev Cell* 8, 267–278 (2005). [PubMed: 15691767]
58. Motzny CK, Holmgren R, The *Drosophila* cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. *Mech Dev* 52, 137–150 (1995). [PubMed: 7577671]

Fig 1. A cell-based RNAi screen identifies Smurf family members as Smo E3 ubiquitin ligases. (A) Family tree of the HECT-domain E3 ubiquitin ligases we targeted by RNAi. (B) Schematic drawings of Smurf, Nedd4, and Su(dx) with the C2, WW, and HECT domains indicated. (C) S2 cells stably expressing Myc-Smo were treated with control dsRNA or dsRNA targeting the indicated HECT-domain E3 ligases. After treatment with the proteasome inhibitor MG132, Myc-Smo was immunoprecipitated (IP) with an anti-Smo antibody, and immunoblotted (IB) with antibodies recognizing ubiquitin or Myc. Loading was normalized by the amount of Myc-Smo. (D) Cell-based ubiquitination assay.

Immunoblot (IB) showing ubiquitination of Myc-Smo immunoprecipitated (IP) from extracts from S2 cells treated with the indicated dsRNAs. Luc dsRNA is a negative control. **(E)** Smo ubiquitination in S2 cells overexpressing Flag (Fg)-tagged Smurf family members Smurf, Nedd4, Su(dx), CG3356, or CG6190. **(F)** Smo ubiquitination in S2 cells overexpressing tagged wild-type (Fg-Smurf) and catalytically inactive (Fg-Smurf^{C1029A}). Immunoblots are representatives of three independent experiments.

Fig 2. The Smurf family of E3s regulates Smo cell surface accumulation in cultured cells
(A) Immunostaining and **(B)** quantification of cell surface Smo in S2 cells stably expressing Myc-Smo and treated with control (Luc) or the indicated dsRNA. Data are mean $\pm$ SD from three independent experiments. $n=10$ cells for each experimental condition. **, $P<0.01$, ***, $P<0.001$ (student's t-test). **(C)** Immunostaining showing a mutant form of Smo that mimics phosphorylation by PKA (Myc-Smo^{SD}) and Fg-Smurf, Fg-Nedd4, or Fg-Su(dx) (Flag) in S2 cells expressing the indicated tagged proteins. **(D)** Quantification of cell surface Myc-Smo^{SD} signals in (C). Data are mean $\pm$ SD from three independent experiments. $n=10$ cells for each experimental condition. ***, $P<0.001$ (student's t-test). **(E)** Immunoblot showing Myc-Smo^{SD} and Fg-Smurf, Fg-Nedd4, or Fg-Su(dx) in S2 cells expressing the indicated combinations of tagged proteins. Myc-CFP is a loading control. The antibody that recognizes GFP also recognizes CFP. **(F)** Quantification of Myc-Smo^{SD} abundance in (E). Data are mean $\pm$ SD from three independent experiments. ***, $P<0.001$ (student's t-test).

(G) Immunoblot showing Smo protein in extracts from cl-8 cells treated with either Hh-conditioned medium (Hh) or dsRNAs targeting Smurf, Neddd4, Su(dx) or all three (3E3s). (H) Quantification of Smo abundance in (G). Data are mean $\pm$ SD from three independent experiments. **, $P<0.01$, ***, $P<0.001$ (student's t-test). (I-J) Quantification of *smo* (I) and *ptc* (J) mRNA by quantitative RT-PCR in cl-8 cells treated with either Hh-conditioned medium or the indicated dsRNAs. (K) Luciferase activity in cl-8 cells expressing the *ptc-luc* reporter gene and treated with either Hh-conditioned medium or the indicated dsRNAs. Data are mean $\pm$ SD from three independent experiments (I-K). Immunofluorescence images are representatives of 10 cells for each condition. Immunoblots are representatives of three independent experiments. Scale bars, 5 μ m.

Fig 3. Smurf reduces Smo cell surface accumulation and Hh signaling in developing wings
(A-D) Late third instar control (ctrl) wing discs (A, C) or wing discs expressing *UAS-Smurf-RNAi* under control of the *MS1096* Gal4 driver (B, D) were immunostained for Ci (red), Smo (green in A and B), or Ptc (green in C and D). Wing discs in these and the following panels are oriented with anterior to the left and ventral up. Arrows and arrowheads indicate the dorsal and ventral compartments of the wing pouch region, respectively. Ci staining marks the anterior compartment cells. **(E-I)** Late third instar control wing disc (E) or wing discs expressing *UAS-Fg-Smurf* under control of the *MS1096* Gal4 driver either alone (F-H)

or together with *UAS-Smurf-RNAi* (I) using *MS1096* were immunostained for Ci (red), Ptc (green in F), En (green in E, G), Smo (green in H, I), or Flag (blue). Dashed lines demarcate the anteroposterior boundary determined by the Ci expression domain (E, G). Arrows indicate the anterodorsal cells near the anteroposterior boundary (E-G) or posterodorsal compartment cells (H-I) of the wing pouch region. Images are representative of five wing discs per genotype. Scale bars, 50 μ m.

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Fig 4. Hh-induced phosphorylation of Smo inhibits Smurf recruitment to Smo. (A) Schematic drawings of full-length Smurf and deletion mutants. (B-C) S2 cells were cotransfected with constructs encoding Fg-Smurf and wild-type Smo (Myc-Smo^{WT}), a deletion mutant lacking the C-tail (Myc-Smo^{CT}), a mutant form of Smo that cannot be phosphorylated by PKA (Myc-Smo^{SA}), or a phosphomimetic form of Smo (Myc-Smo^{SD}) as indicated and treated with control or Hh-conditioned medium (Hh), followed by immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Cells were treated with the proteasome inhibitor MG132 for 4 hours before lysis. Asterisks indicate the monomeric form of full-length (Myc-Smo^{WT}) and truncated (Myc-Smo^{CT}) Smo. (D) S2 cells were cotransfected with Myc-Smo and the indicated deletion forms of Fg-Smurf and subjected to immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Asterisks indicate the full-length and truncated forms of Smurf (D). (E) Cell-based ubiquitin assay showing ubiquitination of Myc-Smo immunoprecipitated from S2

cells coexpressing Myc-Smo and a truncated form of Smurf consisting of only the C-terminal HECT domain (Fg-Smurf-HECT). **(F)** S2 cells were cotransfected with constructs encoding Fg-Smurf-HECT and various forms of Myc-tagged Fz: full-length Fz (Myc-Fz), Fz fused to SAID, the autoinhibitory domain of Smo (Myc-FS), Fz fused to phosphorylation-deficient (Myc-FS^{SA}) or phosphomimetic (Myc-FS^{SD}) forms of the Smo autoinhibitory domain. The Fz proteins were immunoprecipitated (IP) and then immunoblotted (IB) with antibodies recognizing Myc or Fg. **(G)** S2 cells were cotransfected with constructs encoding full-length Fg-Smurf and Myc-FS, Myc-FS^{SA}, or Myc-FS^{SD}. The Myc-tagged proteins were immunoprecipitated (IP) and immunoblotted (IB) with antibodies recognizing Myc or Fg. **(H)** S2 cells with endogenous Smo depleted by dsRNA targeting *smo* 5' UTR were cotransfected with constructs encoding Myc-Smo, Myc-Smo^{SAID}, and Fg-Smurf. The Myc-tagged proteins were immunoprecipitated (IP) and immunoblotted (IB) with antibodies recognizing Myc or Fg. Blots are representatives of three independent experiments.

Fig 5. Gprk2 promotes Smurf recruitment to Smo.

(A-B) Cell-based ubiquitination assay of Myc-Smo in S2 cells treated with Smurf and Gprk2 dsRNAs either alone or in combination (A), or coexpressed with Gprk2 (Fg-Gprk2) or the kinase-dead form Fg-Gprk2^{KM} (B). (C) Immunostaining showing Smo and GFP in late third instar wing discs expressing *UAS-GFP* under the control of the *brk* Gal4 driver. (D-F) Immunostaining showing Smo in late third instar wing discs expressing *UAS-Smurf-RNAi* (D), *UAS-Gprk2-RNAi* (E), or *UAS-Smurf-RNAi + UAS-Gprk2-RNAi* (F) under control of the *brk* gal4 driver. Arrows indicate the anterior *brk* expression domain. (G-I) S2 cells were transfected with the indicated constructs and treated with either control (Luc) dsRNA or dsRNAs targeting the coding sequence (G, I) or 5' UTR (H) of Gprk2, followed by immunoprecipitation (IP) and immunoblotting (IB) with the indicated antibodies. Cells were treated with the proteasome inhibitor MG132 for 4 hours before lysis. Images are representative of five wing discs per genotype. Blots are representatives of three independent experiments.

Fig 6. Gprk2 promotes Smo-Smurf association by phosphorylating Smurf
(A-B) Immunoblots (IB) of lysates from S2 cells expressing Fg-Smurf and Myc-Gprk2 or Myc-Gprk2^{KM} as indicated and treated with control or Hh-conditioned medium (Hh). λ phosphatase (λ -PP) was added to the cell extracts to remove phosphorylation. (C) Immunoblots of lysates from S2 cells expressing Fg-Smurf and Myc-Gprk2 as indicated in the presence of absence of control (Luc) or Smo dsRNA. (D) Immunoblot (IB) showing Fg-Smurf and Myc-Gprk2 in Myc immunoprecipitates (IP) from S2 cells expressing the Myc-Gprk2 and Fg-Smurf and subjected to treatment with Hh-conditioned medium or dsRNAs as

indicated. **(E-F)** Western blot of immunoprecipitates from S2 cells expressing the indicated combinations of the N-terminus of Smurf (Fg-Smurf^{HECT}), a form of Smurf^{HECT} in which the Gprk2 phosphorylation site was mutated (Fg-Smurf^{HECT-SA}), or the HECT domain of Smurf (Fg-Smurf-HECT) with wild-type (Myc-Gprk2) and kinase-dead (Myc-Gprk2^{KM}) forms of Gprk2. **(G)** Western blot of immunoprecipitates from S2 cells expressing full-length Smurf (Fg-Smurf) or a mutant form of Smurf in which the Gprk2 phosphorylation site was mutated (Fg-Smurf^{SA}) with wild-type (Myc-Gprk2). Wild-type sequence of the N-terminal Ser cluster and its SA or SD substitutions are shown in (G). **(H)** Immunoblot showing Smurf and Smo in immunoprecipitates from cells expressing Myc-Smo and the indicated forms of Smurf. **(I)** Immunoblot showing ubiquitination of Smo in S2 cells expressing Myc-Smo and the indicated forms of Smurf. **(J)** Immunoblot showing Smurf and Smo in immunoprecipitates from cells expressing Myc-Smo and the indicated forms of Smurf and treated with control (Luc) or Gprk2 dsRNAs as indicated. **(K-L)** Immunoblot analysis of immunoprecipitates (IP) from cells expressing the indicated proteins. Note that Fg-Gprk2 and Fg-Smurf^{HECT} or Fg-Smurf^{HECT-SA} overlapped on Western blot. Blots are representatives of three independent experiments.

Fig 7. Hh promotes Ptc ubiquitination by dissociating Smurf from Smo and facilitating its binding to Ptc.
(A) Ptc ubiquitination assay in S2 cells treated with control or Hh-conditioned medium (Hh) in the presence of Luc, Smo, Smurf, or both Smo and Smurf dsRNA. **(B)** Immunoblotting (IB) or immunoprecipitates (IP) from S2 cells coexpressing Myc-Ptc and Fg-Smurf and treated with Hh-conditioned medium in the presence of absence of the indicated dsRNAs. **(C-D)** *L>Ptc-GFP* expression in control wing discs **(C)** or wing discs containing clones expressing *UAS-Smo-RNAi* under the control of *act-Gal4* **(D)**. Smo depletion decreased Ptc-

GFP in anterior compartment cells away from the anteroposterior boundary (arrows in D) but not in posterior compartment cells (arrowheads in D). Images are representative of five wing discs per genotype. **(E-F)** Ptc-Smurf association (E) or Ptc ubiquitination (F) in S2 cells expressing the indicated constructs in the presence of Luc or Smo dsRNA. **(G-H)** Ubiquitination of Myc-Ptc or Myc-Ptc^{L2} in S2 cells treated with control or Hh-conditioned medium in the presence of Luc or Ptc dsRNA (G) or in the presence of both Smo and Ptc dsRNA (H). **(I)** Smurf-Ptc (Ptc^{L2}) association in S2 cells treated with the indicated dsRNA and with control or Hh-conditioned medium. Blots are representatives of three independent experiments.

Fig. 8. Model for how Hh regulates the reciprocal trafficking of Smo and Ptc through Smurf
 In the absence of Hh (left), Gprk2-mediated phosphorylation of Smurf promotes Smurf association with Smo, leading to Smo ubiquitination, internalization, and degradation, and Ptc is modestly ubiquitinated by Smurf. In the presence of Hh (right), Smo recruits PKA, which phosphorylates the Smo C-tail, thus inhibiting Smurf binding to and ubiquitinating Smo. Hh promotes the binding of released Smurf to Ptc, leading to increased Ptc ubiquitination and degradation. The different shapes of unphosphorylated and phosphorylated Smurf represent open (ovals) and closed (circles) conformations.