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Commentary
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Regulation of Smoothened Trafficking in Hedgehog Signal Transduction

Shuang Li [1] **and Jin Jiang**[1,2*]

[1]*Department of Molecular Biology, University of Texas, South-Western Medical Center at Dallas, Dallas, Texas 75390, USA*

[2]*Department of Pharmacology, University of Texas, South-Western Medical Center at Dallas, Dallas, Texas 75390, USA*

[*]**Corresponding author:** Jin Jiang, Department of Molecular Biology, University of Texas, South-western Medical Center at Dallas, Dallas, Texas 75390, USA, Tel: 214 645 5914; E-mail: jin.jiang@utsouthwestern.edu

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Commentary

This commentary aims to discuss the findings of our recently published articles in which we identified the Smurf family of HECT-domain containing E3 ubiquitin ligases and the Cul4-DDB1-Gb ubiquitin ligase complex as new regulators of Smoothened (Smo) trafficking and cell surface expression [1,2]. These findings provide novel insights into how Hedgehog (Hh) signaling is regulated. Cell-cell communication mediated by the Hh family of secreted proteins plays central roles in embryonic development and adult tissue homeostasis [3,4]. Deregulation of Hh signaling has been implicated in a wide range of human disorders including birth defects and cancers [3,5]. Hh signaling regulates cell growth and proliferation, cell fate determination, and pattern formation through a conserved signal transduction cascade that culminates in the activation of latent transcription factors Cubitus interruptus (Ci) and Gli proteins [3,6]. The core signal reception system of the Hh pathway constitutes two multi-span transmembrane proteins: a twelve-transmembrane protein Patched (Ptc), which functions as the Hh receptor, and a seven-transmembrane protein Smo, which is a member of the G protein coupled receptor (GPCR) family and functions as the obligated Hh signal transducer. Binding of Hh to Ptc releases its inhibition of Smo, leading to phosphorylation and activation of Smo [7]. Smo is regulated through two paralleled mechanisms: 1) change of its conformation, and 2) change in its subcellular localization. In *Drosophila*, Ptc inhibits Smo phosphorylation, which keeps Smo in an inactive conformation and prevents Smo cell surface accumulation. Upon Hh stimulation, Smo is phosphorylated by protein kinase A (PKA) and casein kinase 1 (CK1), which promotes Smo cell surface accumulation and triggers a conformational change of Smo into an open and active conformation [8-10]. In addition, Hh binding to Ptc facilitates internalization of the Hh-Ptc protein complex, a process required for restricting Hh signaling range [11,12]. Recent studies have revealed that ubiquitination and subsequent degradation of Smo *via* both proteasome and lysosome-dependent mechanisms are responsible for preventing Smo cell surface accumulation, and that Hh-induced phosphorylation of Smo inhibits its ubiquitination and degradation [13,14]. However, the mechanism by which phosphorylation inhibits Smo ubiquitination has remained a mystery due to the lack of information on E3 ubiquitin ligase (s) that targets Smo. Previous genetic screen as well as cell-based RNAi screen failed to identify any E3 that targets Smo for ubiquitination. A likely reason is that multiple E3 ligases could be involved in the regulation of Smo ubiquitination and their functional redundancy could have masked any Hh-related phenotypes when individual E3s were inactivated. In addition, Smo E3s might not be dedicated for the Hh pathway so that their inactivation could cause pleiotropic defects due to dysregulation of multiple protein substrates. We decided to carry out a cell-based RNAi screen using Smo ubiquitination as a sensitive readout [1]. We initially focused on the HECT family of E3 ubiquitin ligases because members in this family have been implicated in the regulation of GPCR trafficking [15]. From this screen, we identified the Smurf subfamily of HECT-domain containing E3s including Smurf, Nedd4, and Su (dx) as Smo ubiquitin ligases [1]. Knockdown of Smurf by RNAi decreased Smo ubiquitination and increased its cell surface abundance in S2 cells, a phenotype enhanced by simultaneous knockdown of Nedd4 and Su (dx) in conjunction with Smurf knockdown. On the other hand, overexpression of Smurf, Nedd4, or Su (dx) promoted Smo ubiquitination and prevented its cell surface accumulation. Co-immunoprecipitation experiments showed that Smurf binds Smo C-terminal tail (Smo-CT) through its HECT domain in the absence of Hh; however, in response to Hh stimulation, PKA/CK1-mediated phosphorylation of Smo dissociates Smurf from Smo-CT, thereby inhibiting Smo ubiquitination (Figure 1) [1].

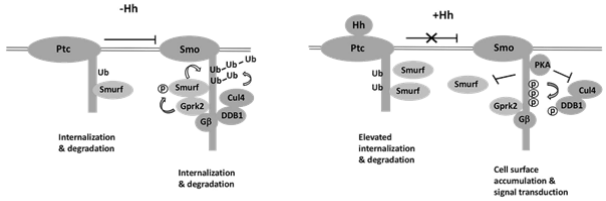

Figure 1: Regulation of Smo trafficking and cell surface expression by multiple E3 ubiquitin ligases.

Interestingly, we found that the N-terminal region of Smurf binds to its C-terminally localized HECT domain to prevent Smurf from binding to Smo, and that G protein coupled receptor kinase 2 (Gprk2)-mediated phosphorylation of the N-terminal region alleviates this auto-inhibition and frees the HECT domain for Smo binding [1]. Loss of Gprk2 compromised the recruitment of Smurf to Smo-CT and as a consequence, the ubiquitination of Smo [1]. This explains the previous observation that Smo was upregulated in Gprk2 mutant wing imaginal discs [10,16]. A previous study revealed that Smurf also regulates Ptc ubiquitination and internalization [17]. Interestingly, we found that Ptc and Smo compete for the same pool of Smurf family of E3s, and that Hh promotes Ptc ubiquitination by releasing Smurf family members from Smo and further stimulating their binding to Ptc in a manner depending on Hh/Ptc interaction (Figure 1) [1]. Hence, by employing the same set of E3 ubiquitin ligases to reciprocally regulate the trafficking of Ptc and Smo, Hh can effectively coordinate the activities of its receptor and signal transducer. Knockdown of Smurf in cultured S2 cells or *Drosophila* wing imaginal discs only resulted in a mild increase of Smo cell surface expression [1]. Aside from the redundant

function of Need4 and Su (dx), additional E3 (s) could participate in the regulation of Smo cell surface abundance. Indeed, in a paralleled RNAi screen in wing imaginal discs using Smo immunostaining as a readout, we identified the Cullin family member Cullin4 (Cul4) as another regulator of Smo cell surface abundance [2]. Cul4 forms multi-subunit E3 ubiquitin ligase complexes called CRL4s, which contain Cul4, DNA damage-binding protein 1 (DDB1), one of the DDB1-binding WD40 family proteins (DWD) that recognize protein substrates of CRL4s [18]. We found that knockdown of Cul4-DDB1 by RNAi resulted in cell surface accumulation of Smo in anterior compartment cells of the wing imaginal discs as well as in S2 cells [2]. Knockdown of Cul4-DDB1 reduced whereas overexpression of Cul4 promoted Smo ubiquitination in S2 cells. We found that Cul4-DDB1 is recruited to Smo-CT through multiple G protein b subunits (Gb) including Gb13F and Gb76C, which are DWD proteins, and that Hh inhibits Cul4-mediated ubiquitination of Smo by recruiting PKA to phosphorylate DDB1, leading to the dissociation of Cul4-DDB1 from Smo-Gb (Figure 1) [2,19]. Simultaneous inactivation of Smurf and Cul4-DDB1-Gb resulted in a more dramatic accumulation of Smo on the surface of S2 cells than inactivation of either Smurf or Cul4-DDB1-Gb alone [1,2], suggesting that Cul4-DDB1-Gb acts in parallel with the Smurf family of E3s to regulate Smo trafficking and abundance (Figure 1). A previous study has revealed that Cul4-DDB1-Gb regulates GPCR signaling by binding to and targeting Gprk2 for ubiquitination [20]. Taken together, these observations suggest that the Cul4-DDB1-Gb E3 ubiquitin ligase may play a broad role in the regulation of membrane receptor trafficking and signaling, a possibility that should be further explored in the future.

Regulation of E3 recruitment by phosphorylation may only account for part of the mechanisms that regulate Smo trafficking and cell surface abundance. Indeed, our recent study has revealed that sumoylation of Smo recruits a deubiquitinase UBPY/USP8 to inhibit Smo ubiquitination, thereby promoting its cell surface accumulation [21]. Taken together, our studies have raised a question of why Smo is regulated by so many paralleled mechanisms. 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. Therefore, it would be interesting to determine whether different mechanisms respond differentially to different levels of Hh signal. It would be also interesting to determine whether different mechanisms are employed in tissue- or context-dependent manners. Finally, we provided evidence that Sonic Hedgehog (Shh) also stimulates sumoylation of mammalian Smo (mSmo) to facilitate its ciliary accumulation [21]; however, the underlying mechanism has remained unexplored. It would be interesting in the future to determine whether Smurf or Cul4 family of E3s regulates mSmo ubiquitination and ciliary trafficking, and whether sumoylation of mSmo promotes its ciliary accumulation by antagonizing ubiquitination.

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