

Article

E3 ligase Herc4 regulates Hedgehog signalling through promoting Smoothened degradation

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Hedgehog (Hh) signalling plays conserved roles in controlling embryonic development; its dysregulation causes many diseases including cancers. The G protein-coupled receptor Smoothened (Smo) is the key signal transducer of the Hh pathway, whose posttranslational regulation has been shown to be critical for its accumulation and activation. Ubiquitination has been reported an essential posttranslational regulation of Smo. Here, we identify a novel E3 ligase of Smo, Herc4, which binds to Smo, and regulates Hh signalling by controlling Smo ubiquitination and degradation. Interestingly, our data suggest that Herc4-mediated Smo degradation is regulated by Hh in PKA-primed phosphorylation-dependent and independent manners.

Keywords: Hedgehog, Smoothened, Herc4, ubiquitination

Introduction

Hedgehog (Hh) signalling plays essential roles in embryonic development and adult tissue homeostasis in species ranging from insects to human (Ingham and McMahon, 2001; Lum and Beachy, 2004; Jia and Jiang, 2006; Jiang and Hui, 2008). Aberrant Hh signalling activity is associated with many human disorders including birth defects and cancers (Taipale and Beachy, 2001; Pasca di Magliano and Hebrok, 2003; Jiang and Hui, 2008; Ingham et al., 2011).

Hh transduces signal through binding to its receptor, a 12-transmembrane protein Patched (Ptc), which alleviates Ptc-mediated suppression of Smo, a seven-transmembrane GPCR protein (Hooper and Scott, 1989; Marigo et al., 1996; Stone et al., 1996; Casali and Struhl, 2004; Corbit et al., 2005; Rohatgi et al., 2007; Zhao et al., 2007). Activated Smo recruits the kinesin-like protein Costal2 (Cos2) and the Ser/Thr kinase Fused (Fu) to trigger the activation of latent transcription factor Cubitus interruptus (Ci) (Alcedo et al., 1996; Sisson et al., 1997; Deneff et al., 2000; Jia et al., 2003), which enters the nucleus to turn on the expression of Hh target genes including *decapentaplegic*

(*dpp*), *patched* (*ptc*), and *engrailed* (*en*) (Ingham and McMahon, 2001; Jia et al., 2005; Smelkinson et al., 2007; Lv et al., 2018a, b).

Protein turnover mediated by ubiquitin modification plays important roles in the regulation of numerous cellular processes during development. Ubiquitination is an enzymatic process by which proteins are modified with ubiquitin chains (Hochstrasser, 1995). This process of delivering ubiquitin to substrates requires the coordinated action of three classes of enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin-protein ligase (Hochstrasser, 1992). While E1 and E2 enzymes are primarily involved in activating and transferring ubiquitin through high-energy thioester bonds, E3 ligases play crucial roles in providing the specificity of substrate recognition (Hochstrasser, 1996). Ubiquitin ligases (E3) have been grouped into two families: the single-subunit forms including single RING-finger, U-box, and HECT-domain ligases (Qiu et al., 2000) and the multi-subunit RING-finger ubiquitin ligases, including the Cullin-Rbx type (Liu and Nussinov, 2011) and the APC E3 ligases (Huang et al., 2005).

The E3 ubiquitin ligases targeting the core components of Hh pathway have been mostly identified. However, the molecular mechanism underlying Smo degradation remains largely unknown. In this study, we identify the HECT E3 ubiquitin ligase Herc4 that compromises Hh signalling by directly controlling Smo protein turnover. Moreover, we find that Herc4 mediates Smo degradation in a manner that is regulated by Hh.

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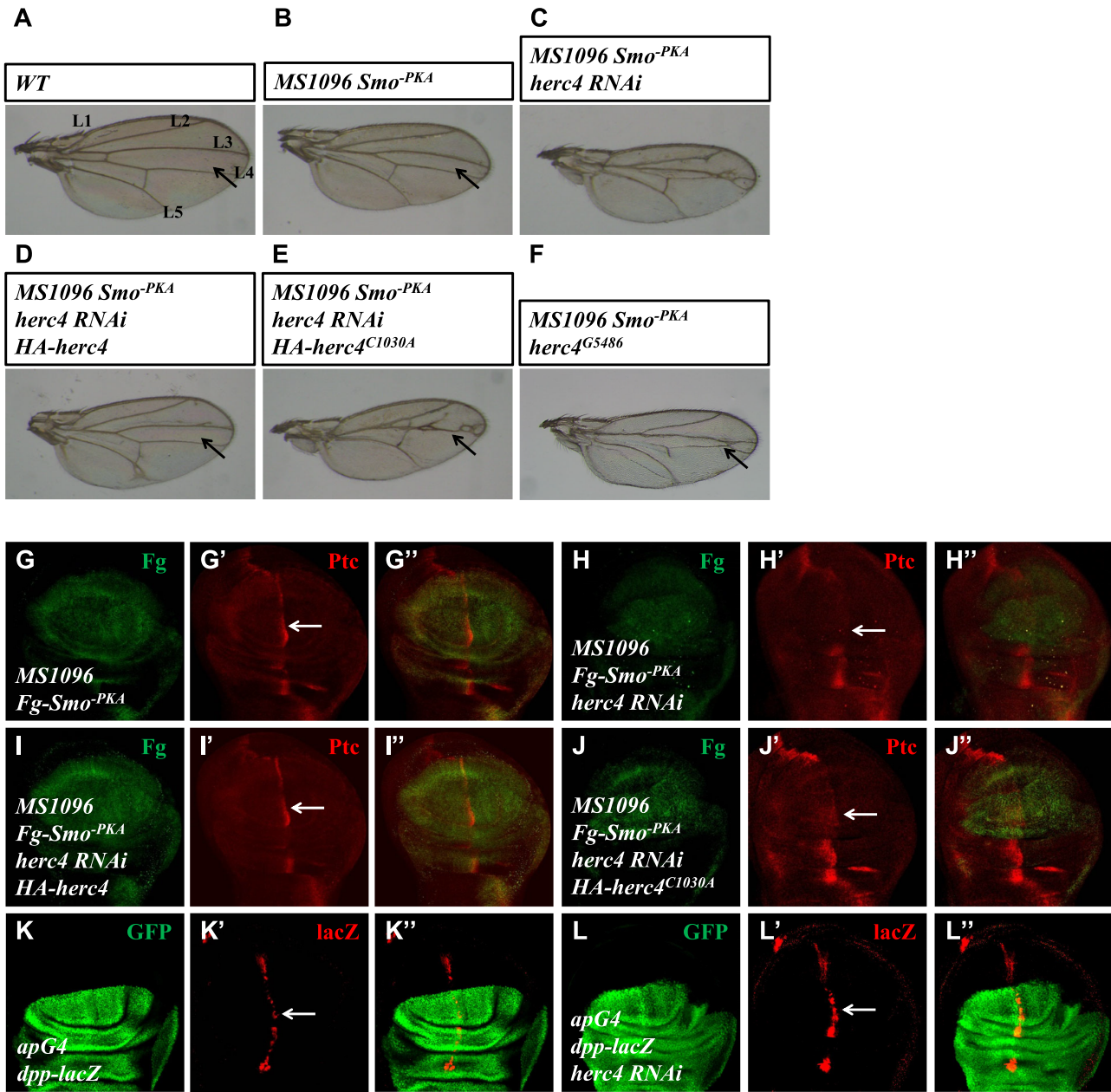


Figure 1 Knockdown of *herc4* upregulates Hh signalling activity. **(A and B)** Comparison of adult wing phenotypes between control **(A)** and *Smo^{-PKA}* overexpression flies **(B)**. Arrows indicate the space between veins 3 and 4. **(C and D)** Overexpression of *Herc4* **(D)** rescued the *herc4* knockdown **(C)**-caused adult wing phenotype under the background of *Smo^{-PKA}*. **(E)** Overexpression of *Herc4^{C1030A}* enhanced the *herc4* knockdown-caused adult wing phenotype under the background of *Smo^{-PKA}*. Arrows indicate the space between veins 3 and 4. **(F)** The mutant of *herc4*, *herc4^{G5486}*, showed *herc4* RNAi-like but weak wing phenotypes in expression of *Smo^{-PKA}* background (compare **F** with **C**). **(G–H'')** Knockdown of *herc4* with *MS1096-Gal4* under the background of *Smo^{-PKA}* attenuated the expression of *Ptc* (compare **H–H''** with **G–G''**). Arrows indicate the decrease of *Ptc*. **(I–I'')** Overexpression of *HA-Herc4* rescued the *herc4* knockdown-caused *Ptc* expression level under the background of *Smo^{-PKA}*. **(J–J'')** Overexpression of *Herc4^{C1030A}* did not rescue the *herc4* knockdown-caused *Ptc* expression level under the background of *Smo^{-PKA}*. **(K–L'')** *UAS-GFP* (green) marks the *apG4*-mediated gene expression region. *apG4* drives *UAS* transgenes to be specifically expressed in the dorsal region of wing discs. Compared with the control disc **(K–K'')**, knockdown of *herc4* by *apG4* upregulated the expression of *dpp-lacZ* **(L–L'')** (arrow).

Results

Inactivation of herc4 positively regulates Hh pathway activity

To identify novel Hh signalling regulators, we carried out a genetic modifier screen in the expression of a dominant-negative Smo (Smo^{-PKA}) background with a wing-specific *Gal4* driver *MS1096* and determined which genes modulated Smo^{-PKA} wing phenotype (Figure 1A and B). From this screen, we identified that knockdown of HECT domain E3 ligase gene *herc4* (CG9153) enhanced *MS1096* > Smo^{-PKA}-induced wing phenotype (compare Figure 1C with B). To exclude the possible off-target effect of RNAi, we coexpressed HA-Herc4 transgene with the corresponding *herc4* RNAi and found that the wing phenotype caused by *herc4* RNAi was totally rescued (compare Figure 1D with C). In addition, the mutant of *herc4*, *herc4*^{G5486}, also showed *herc4* RNAi-like but weak wing phenotype (compare Figure 1F with B). All these results indicate that the wing phenotype caused by downregulation of *herc4* is not due to the off-target effect.

To determine whether Herc4 modifies Hh signalling activity, we examined Hh target genes expression in wing discs. Compared with control discs (Figure 1G–G’), knockdown of *herc4* with *MS1096-Gal4* apparently reduced Ptc level under Smo^{-PKA} background (Figure 1H–H’). However, interestingly, when using a stronger *gal4* driver *ap-Gal4* (*apG4*) to knock down *herc4* in the dorsal region of wing discs in wild-type background, we found that knockdown of *herc4* elevated *dpp-lacZ* expression (Figure 1K–L’). The explanation of this obvious opposite wing phenotypes is that most possibly the substrate of the E3 ligase Herc4 is Smo. In Smo^{-PKA} background, knockdown of *herc4* caused Smo^{-PKA} accumulation, which masked the endogenous smo accumulation effect, at last caused the reduced Ptc level, however, in wild-type background, knockdown of *herc4* caused endogenous smo accumulation which is consistent with the increased *dpp* level. Taken together, these results suggest that Herc4 is a negative regulator of Hh pathway.

In addition, through rescue assays, we found that wild-type Herc4 restored the wing phenotype induced by *herc4* RNAi (Figure 1D and I–I’). By contrast, overexpression of a catalytically dead form of Herc4 (Herc4^{C1030A}) (Aerne et al., 2015) deteriorated the phenotype caused by *herc4* RNAi (Figure 1E and J–J’), indicating that Herc4 plays the function in the E3 ligase activity dependent manner and Herc4^{C1030A} has a dominant-negative effect.

Herc4 regulates Hh signalling through Smo

To verify our hypothesis that Smo is the target of Herc4, we first checked the subcellular localization of Herc4, found Herc4 was localized in both cytoplasm and nucleus (Supplementary Figure S2). Next, we did the co-IP experiments in S2 cells, and found that Herc4 interacted with Smo (Figure 2A). Except this, we did not observe any interaction between Herc4 and other indicated components of Hh pathway, including Ci, Fu, Cos2, PKA-C1 (Supplementary Figure S1A–D).

Herc4 contains two distinct domains: the RCC repeat domain, which is a seven-bladed propeller-like domain, and the HECT

domain, which is involved in regulating the E3 ligase activity for ubiquitination (Figure 2B). To map the domains required for Herc4 binding to Smo, we carried out co-IP experiments using truncated fragments of Herc4. Through co-IP experiments, we found either RCC repeat domain or the HECT domain is sufficient to mediate its binding to Smo (Figure 2C–D).

To determine which region of Smo is responsible for binding to Herc4, various Myc-Smo truncated mutants were transfected into S2 cells with HA-Herc4, followed by co-IP assays (Figure 2E). As shown in Figure 2F–G, both N- and C-terminus of Smo (Myc-SmoN and Myc-SmoC) interacted with HA-Herc4. Previous studies have demonstrated that the C-terminally located Smo auto-inhibitory domain (SAID) plays an important role in regulating Smo activity (Zhao et al., 2007; Li et al., 2012). To test whether SAID domain is responsible for SmoC interacting with Herc4, we investigated various SmoC truncations for their ability to bind Herc4. As shown in Figure 2H–I, SAID domain itself could, but SmoC with SAID domain deleted could not bind Herc4, indicating that SAID domain is necessary and sufficient to mediate the interaction between Herc4 and SmoC. Taken together, Herc4 could interact with both N-terminus and C-terminal SAID domain of Smo.

Herc4 regulates Smo stability

Because Herc4 is an E3 ligase and interacts with Smo, we first examined whether Herc4 can regulate Smo level. As shown in Figure 3A, overexpression of Flag-Herc4 (Fg-Herc4) in S2 cells apparently downregulated Myc-Smo protein level, while knockdown of *herc4* increased its level. Consistently, overexpression of Fg-Herc4 in S2 cells markedly decreased the cell membrane localized Myc-Smo level (Figure 3B–C’). Next, we explored the effect of Herc4 on Smo *in vivo*. Knockdown of *herc4* in wing imaginal discs with *apGal4* promoted Smo accumulation in anterior-dorsal compartment cells (Figure 3D–D’). Of note, *herc4* knockdown did not change the *Smo* mRNA levels (Figure 3E), suggesting that Smo is regulated by Herc4 through a post-translational mechanism.

To determine whether Herc4 regulates Smo at protein level, we carried out a protein stability assay. S2 cells were transfected with Myc-Smo and Fg-Herc4, then, treated with cycloheximide (CHX) to block protein synthesis for indicated times. The results showed that overexpression of Fg-Herc4 apparently promoted Smo degradation (Figure 3F). While overexpression of HA-Herc4^{C1030A} markedly decreased Smo degradation (Figure 3G). These results suggest that Herc4 negatively regulates Hh signalling by binding and degrading Smo.

Herc4 promotes Smo degradation mainly through SAID domain

From above results we have known that Herc4 regulates Hh signalling through its ubiquitinase activity. Next we examined whether Herc4 ubiquitinates Smo. Via cell-based ubiquitination assay, we found overexpression of the wild-type Herc4 significantly increased Smo ubiquitination, whereas the Herc4^{C1030A} decreased Smo ubiquitination (Figure 4A). Furthermore, as

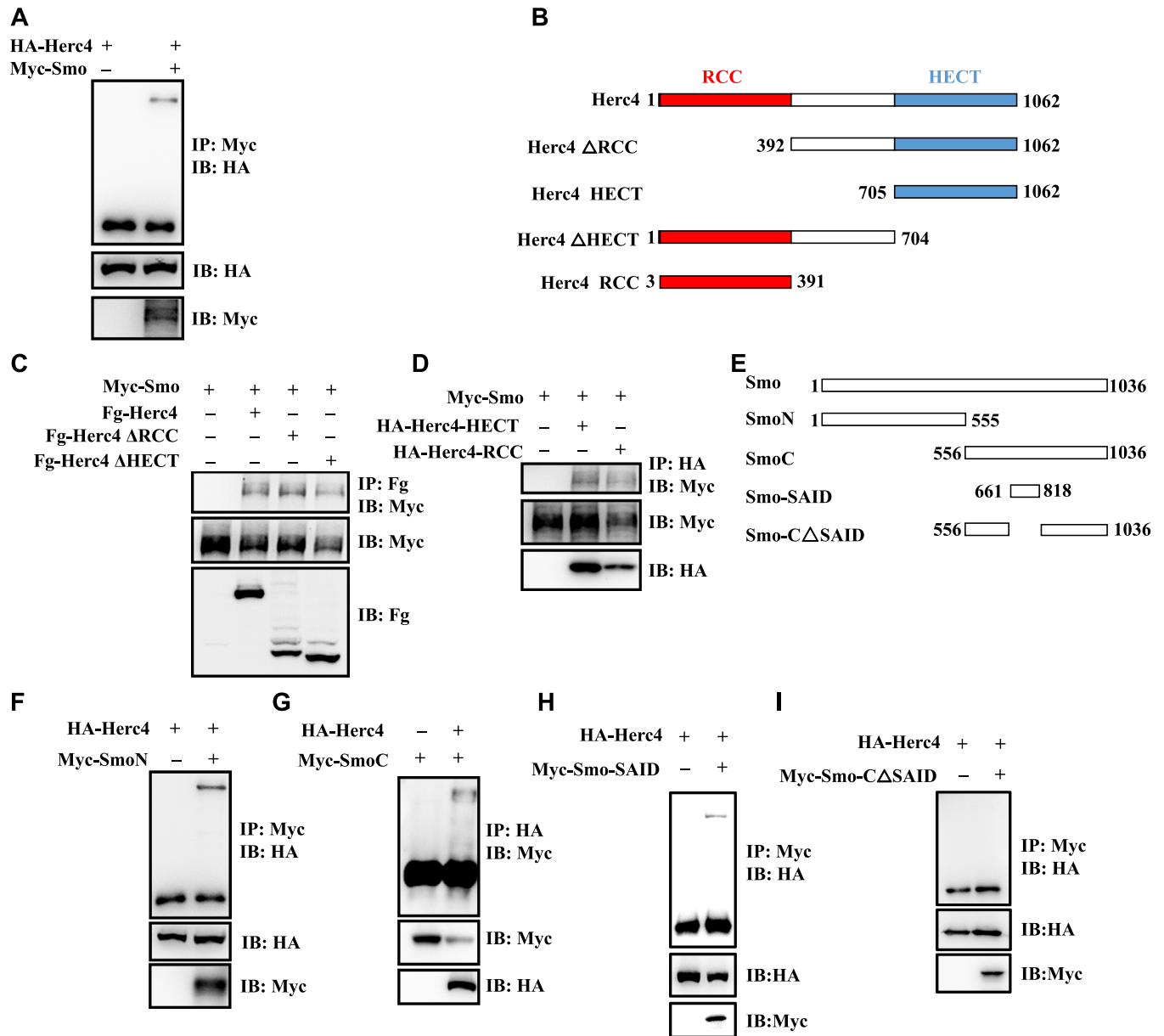


Figure 2 Herc4 binds with Smo. (A) HA-Herc4 interacted with Myc-Smo. (B) Schematic drawings show the domains of Herc4. (C) Both the Herc4 Δ RCC and Herc4 Δ HECT interacted with Smo in S2 cells. (D) Both the Herc4-RCC and Herc4-HECT interacted with Smo in S2 cells. (E) Schematic drawings show the various Smo constructs. (F–H) Herc4 bound with the N-terminus (F), C-terminus (G), and the SAID domain (H) of Smo. (I) Herc4 did not bind with Myc-Smo-C Δ SAID.

shown in Figure 4B, knockdown of *herc4* resulted in a marked reduction of Smo ubiquitination compared to control *gfp* RNAi treatment. The efficiency of *herc4* RNAi was confirmed by semi-quantitative PCR analysis.

To establish which part of Smo mediates its ubiquitination, we analysed SmoN and SmoC. As shown in Figure 4C, Smo ubiquitination occurred mainly within SmoC. To further analyse which domain of SmoC mediates its ubiquitination, we generated SmoC1, SmoC2, and SmoC Δ SAID truncated forms (Figure 4D). As shown in Figure 4E and F, overexpression of

Herc4 significantly decreased the protein level and increased the ubiquitination of SmoC1, which comprising SAID domain, but had no effect on SmoC2 and SmoC Δ SAID, indicating SAID domain mediated SmoC ubiquitination and degradation. Consistently, as shown in Figure 4G–I, Herc4 obviously promoted the SAID domain ubiquitination and degradation, but failed to degrade SAID-deleted full length Smo (Myc-Smo Δ SAID). Taken together, the results support that Herc4 promotes Smo ubiquitination and degradation mainly through its SAID domain.

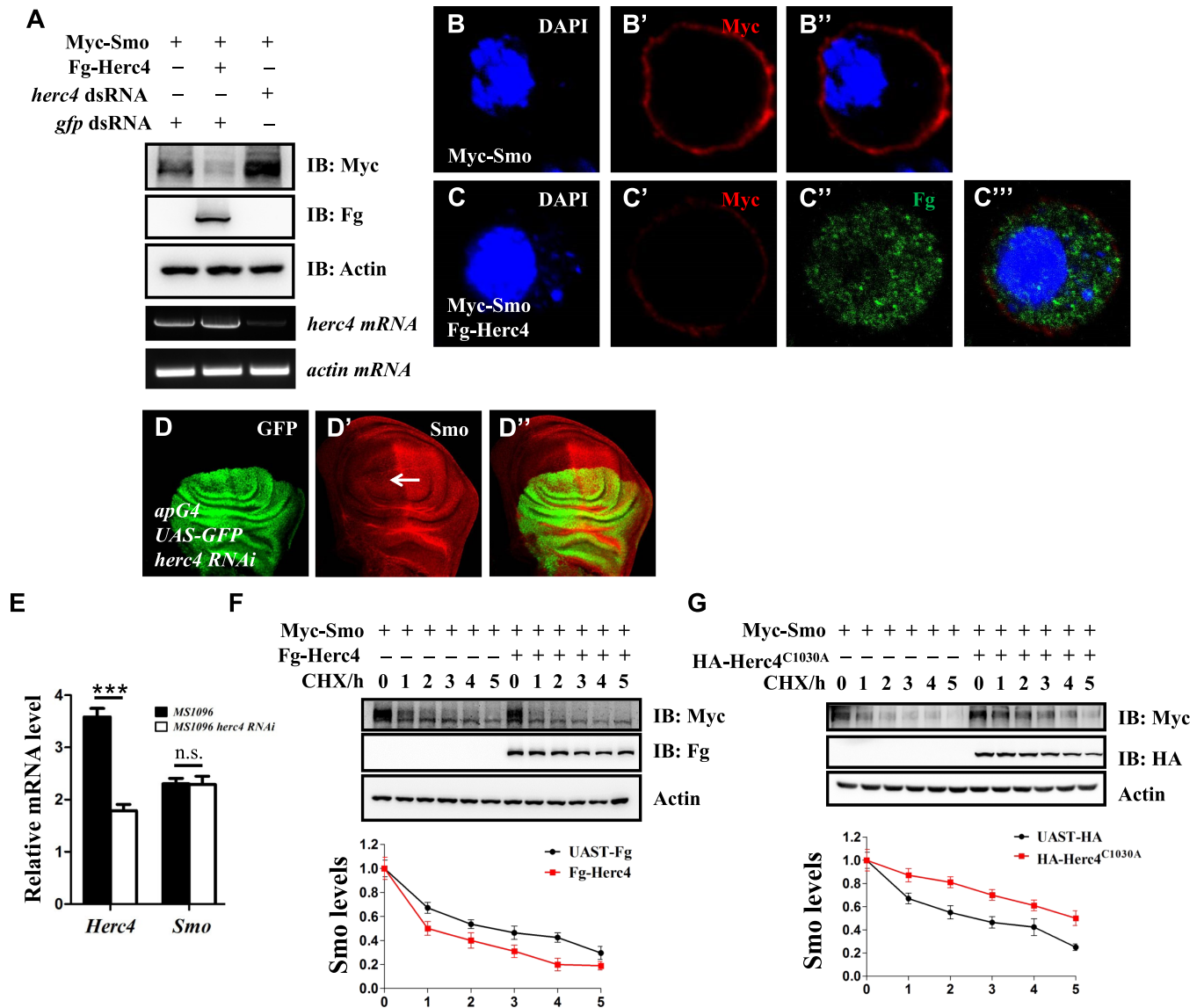


Figure 3 Herc4 affects Smo stability. (A) Overexpression of Fg-Herc4 downregulated Myc-Smo protein level. Knockdown of *herc4* upregulated Myc-Smo protein. *herc4*-dsRNA could effectively knock down *herc4* mRNA level in S2 cells (bottom two panels). (B–C'') S2 cells transfected with indicated constructs were stained by Myc, Flag antibody and DAPI. Of note, Herc4 inhibited Smo cell membrane accumulation (compare C–C'' with B–B''). The nuclei were showed by DAPI staining. (D–D'') Knockdown of *herc4* with *apG4* increased the anterior compartment Smo protein level of the wing disc. Arrows indicate the increase of Smo. (E) The relative mRNA level of *smo* in wing discs. (F and G) Western blots of lysates from S2 cells expressing indicated proteins and treated with CHX for the indicated time intervals. Quantification analyses were shown below. The results were presented as mean \pm SD of values from three independent experiments. Of note, Herc4 could promote Smo degradation (F). Herc4^{C1030A} could hamper Smo degradation (G).

Herc4 promotes Smo degradation through both lysosome and proteasome

Previous studies have demonstrated that Smo is degraded in both proteasome and lysosome (Molnar et al., 2011; Li et al., 2012; Xia et al., 2012). To evaluate their roles in Herc4-mediated Smo degradation, we overexpressed Myc-Smo with Fg-Herc4 in S2 cells and treated the cells with NH₄Cl (lysosome

inhibitor) or MG132 (proteasome inhibitor). We found that treating S2 cells with NH₄Cl or MG132 stabilized Herc4-mediated Smo, and the combined treatment of cells with MG132 and NH₄Cl had an additive effect on Herc4-mediated Smo stabilization. However, MG132 treatment resulted in a marked inhibition in Herc4-mediated Smo degradation compared with treatment with NH₄Cl (Figure 5). These results indicate that Smo protein

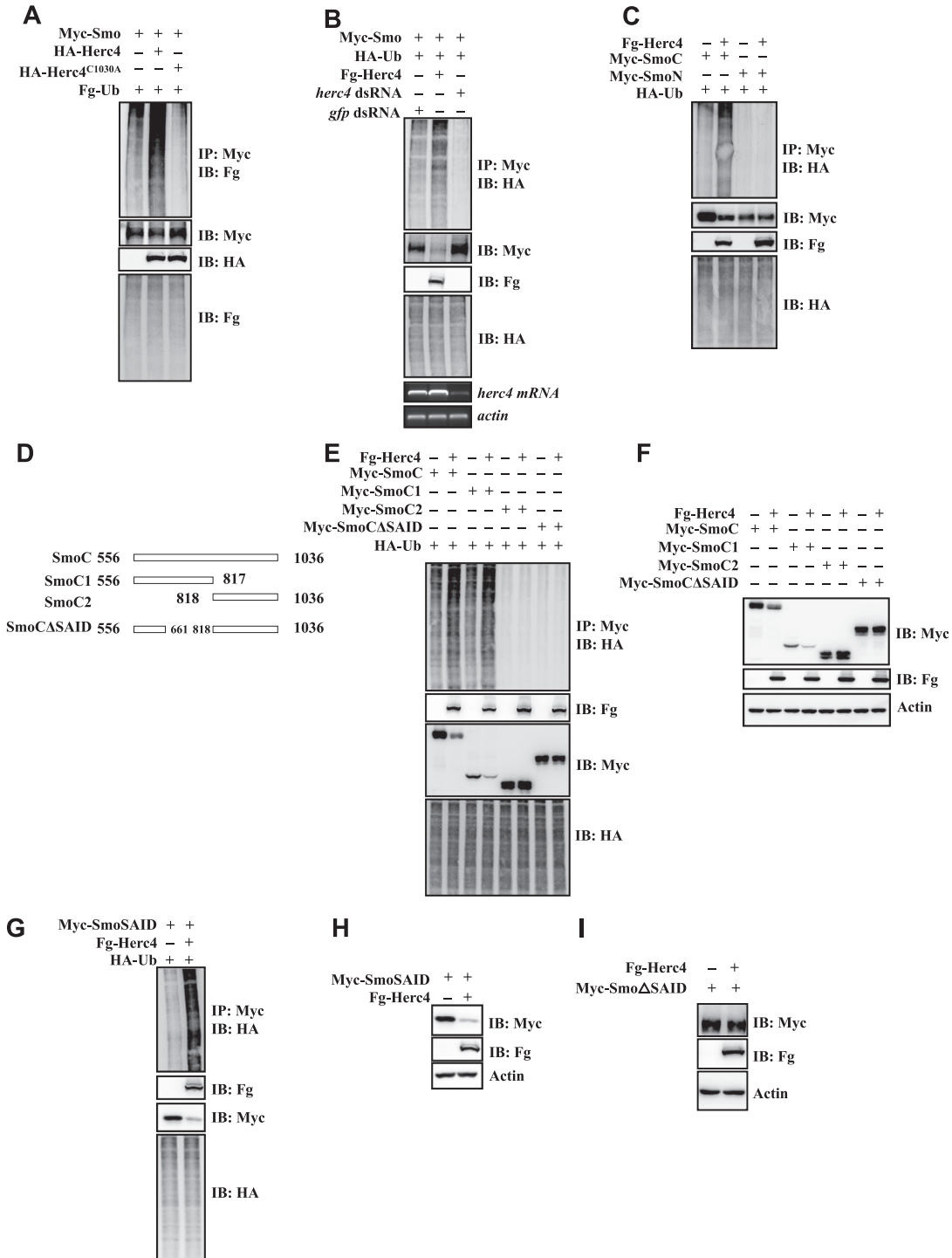


Figure 4 Herc4 promotes Smo degradation mainly through SAID domain. **(A)** S2 cells transfected with the indicated plasmids were examined by western blotting. Of note, HA-Herc4 increased, but HA-Herc4^{C1030A} decreased Smo ubiquitination. **(B)** Knockdown of *herc4* using *herc4*-dsRNA attenuated Smo ubiquitination in S2 cells. *herc4*-dsRNA could effectively knockdown *herc4* mRNA level in S2 cells (bottom two panels). **(C)** S2 cells transfected with indicated plasmids were examined by western blotting. Of note, Fg-Herc4 mainly ubiquitinated SmoC to promote Smo degradation. **(D)** Schematic drawings show the truncated forms of SmoC. **(E)** S2 cells were transfected with the indicated plasmids. The western blot result showed that Herc4 mainly regulated the ubiquitination level of SmoSAID domain. **(F)** The truncated forms of SmoC were transfected alone or with Herc4 into S2 cells. The results showed Herc4 degraded SmoC through its SAID domain. **(G)** Fg-Herc4 promoted Myc-SmoSAID ubiquitination. **(H)** Overexpression of Fg-Herc4 apparently downregulated the protein level of Myc-SmoSAID. **(I)** Overexpression of Fg-Herc4 hardly downregulated the protein level of Myc-SmoΔSAID.

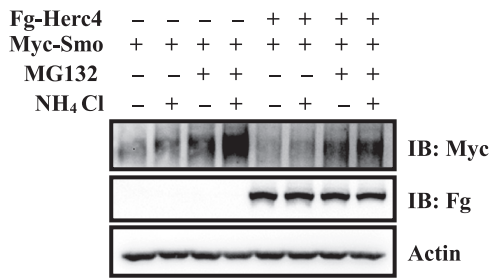


Figure 5 Herc4 degrades Smo mainly through proteasome. S2 cells were transfected with the indicated plasmids, treated with MG132 alone, NH₄Cl alone, or together. The results showed Herc4 degraded Smo mainly through proteasome.

can be degraded through both proteasome and lysosome, but Herc4-mediated Smo degradation is mainly conducted through proteasome.

Herc4 mediates Smo monoubiquitination and polyubiquitylation

Ubiquitin-mediated protein degradation can occur through monoubiquitination or polyubiquitination (Shabek et al., 2012). To test which type of ubiquitination contributes to Smo degradation, we employed Ub-KO, a mutant form of Ub, in which all seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) are replaced by arginines (Rs), preventing polyubiquitin chain formation (Kulathu and Komander, 2012). Compared with wild-type Ub, when Smo co-expressed with Ub-KO, its ubiquitination level apparently decreased (Figure 6A), suggesting that Smo is subjected to polyubiquitination.

We next employed various HA-Ub mutants (Ub-K6R, Ub-K11R, Ub-K27R, Ub-K29R, Ub-K33R, Ub-K48R, and Ub-K63R) to examine the pattern of Smo ubiquitination. Co-transfecting Myc-Smo and Flag-Herc4 together with various HA-Ub mutants, we found the ubiquitination level of Smo was similar to HA-Ub when various HA-Ub mutants were used (Figure 6B and C), indicating that any single type of HA-Ub mutant is not sufficient to block the Smo ubiquitination and Herc4 mediates Smo polyubiquitination with multi-types of ubiquitin chains.

SAID domain harbours a total of 13 Lys residues (K1–K13). We generated two mutant forms of Smo, Myc-SmoK6R and Myc-SmoK7R, in which the K1–K6 and the K7–K13 of SAID domain were mutated to Rs, respectively. Co-expressing HA-UbK0 and Flag-Herc4 together with Myc-SmoK6R or Myc-SmoK7R, we found that Myc-SmoK6R and Myc-SmoK7R were effectively modified by HA-UbK0 (Figure 6D), suggesting that Smo can be monoubiquitinated at multiple sites. Taken together, Herc4 mediates Smo monoubiquitination and polyubiquitylation.

Herc4 mediates Smo ubiquitination at multiple lysine residues

We have known that Herc4 mediates Smo degradation by promoting C-terminal ubiquitination. We speculate that this region might contain Lys residues critical for Smo ubiquitination. Smo C-terminal and intracellular loops contain a total of 49 Lys residues

and there are a total of 13 Lys residues in the SAID domain. Using the cell-based ubiquitination assay, we found that both Myc-SmoK13R and Myc-SmoK49R exhibited reduced ubiquitination compared with Myc-Smo by Herc4, but Myc-SmoK49R resulted in a more dramatic reduction in Smo ubiquitination (Figure 6E), suggesting that Smo is ubiquitinated by Herc4 at multiple Lys residues. In addition, the residual ubiquitination of Myc-SmoK13R suggests that Herc4 mediates Smo ubiquitination at one or more Lys residues outside the SAID domain.

Herc4 interacts with USP8, UCHL5, and Hrs to regulate Smo ubiquitination

USP8 and UCHL5 are Smo deubiquitinases that can decrease the Smo ubiquitination level (Li et al., 2012; Xia et al., 2012; Zhou et al., 2018). To test whether USP8, UCHL5 attenuate Herc4-mediated ubiquitination of Smo, we did the western blot experiments. The results showed that USP8, UCHL5 alone could decrease, they together almost abolished Herc4-mediated Smo ubiquitination (Figure 7A). Interestingly, we found overexpression of Fg-UCHL5 and Fg-USP8 alone decreased the interaction between Herc4 and Smo, simultaneous overexpression of Fg-UCHL5 and Fg-USP8 almost blocked the interaction between Herc4 and Smo (Figure 7B), indicating maybe there are competing binding among them.

The previous study has reported that hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) binds Smo and promotes its ubiquitination (Fan et al., 2013). It is possible that Hrs regulates Smo ubiquitination by facilitating the recruitment of more ubiquitin ligase Herc4 into Hrs/Smo complex. To test this hypothesis, we did a co-IP experiment, and found that Fg-Hrs indeed interacted with HA-Herc4 (Figure 7C), but did not affect the interaction of HA-Herc4 and Myc-Smo (Figure 7D). Furthermore, we found that Hrs-mediated Smo ubiquitination was almost blocked by knockdown of *herc4* (Figure 7E). All these results support the idea that Hrs regulates Smo ubiquitination largely through recruiting Herc4 to Smo.

Herc4-mediated Smo ubiquitination is regulated by Hh

The ubiquitination status of Smo is tightly controlled by the Hh signalling. We next want to test whether the interaction between Smo and Herc4 is regulated by Hh. In S2 cells, co-expressing Myc-Smo and Fg-Herc4 with or without Hh treatment, we found that Hh treatment inhibited the interaction between Smo and Herc4 (Figure 8A) and prevented Herc4-mediated Smo ubiquitination (Figure 8B).

Phosphorylation of Smo is tightly controlled by the Hh signal gradient, and is very important for its activity and cell plasma membrane localization (Jia et al., 2004; Zhao et al., 2007). The activated Smo phosphorylation can be mimicked by SmoSD mutants, in which three PKA sites (Ser667, Ser687, and Ser740) and adjacent CKI sites are mutated to aspartic acid. Another Smo mutant, SmoSA, as mentioned before, which has three PKA sites mutated to alanine, can mimic the situation in which the Smo fails to be phosphorylated and no longer responds to the Hh

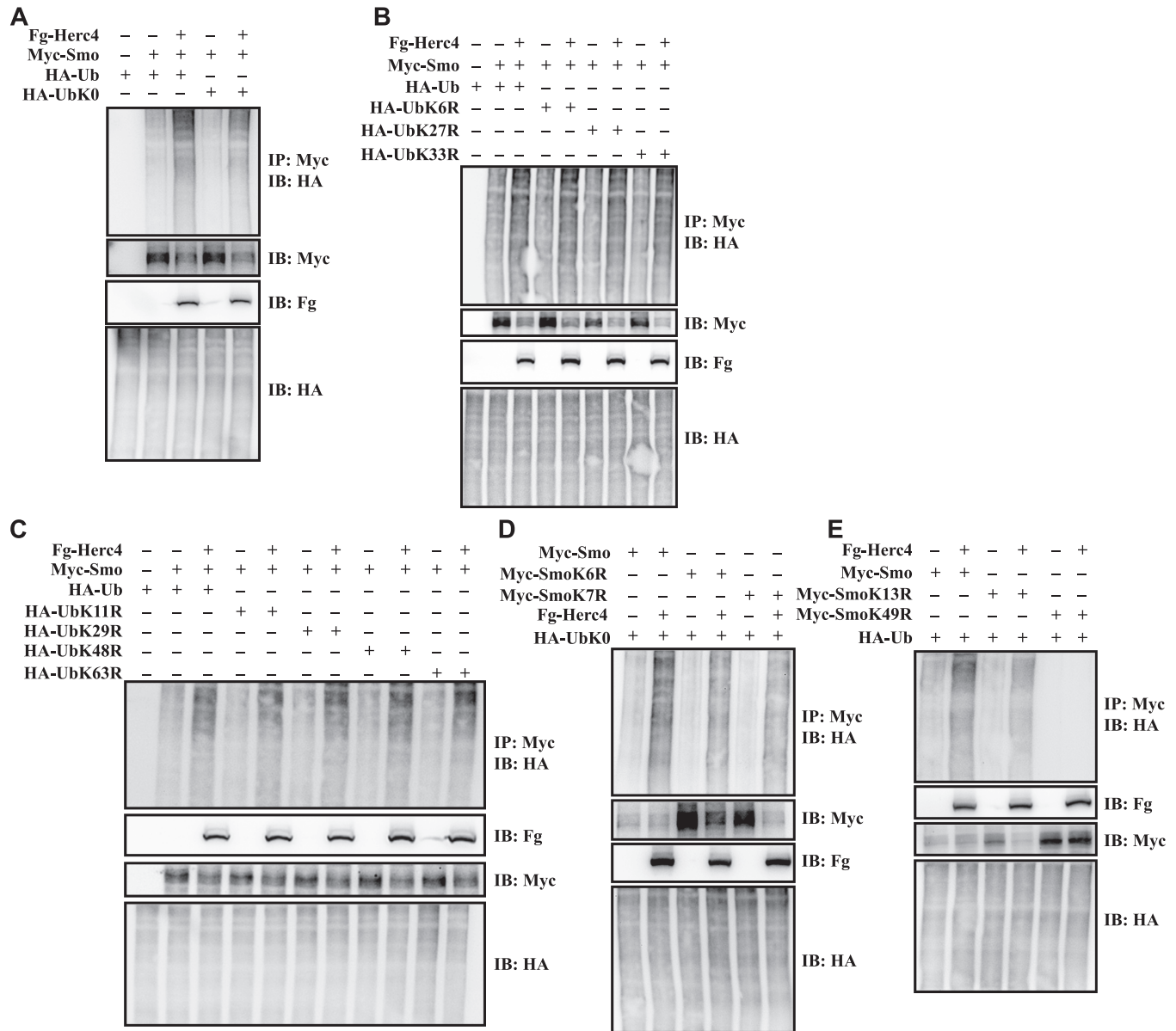


Figure 6 Herc4 mediates both monoubiquitination and polyubiquitylation of Smo ubiquitination. **(A)** S2 cells transfected with the indicated plasmids were examined by western blotting. Of note, the ubiquitination level of Smo apparently decreased when co-expressed with Ub-K0 compared with wild-type Ub. **(B and C)** Co-transfection of Myc-Smo and Flag-Herc4 together with various HA-Ub mutants, the ubiquitination level of Smo was similar to HA-Ub when various HA-Ub mutants were used. **(D)** Co-expression of HA-UbK0 and Flag-Herc4 together with Myc-SmoK6R or Myc-SmoK7R, Myc-SmoK6R, and Myc-SmoK7R were effectively modified by HA-UbK0. **(E)** Herc4 ubiquitinated Smo through multiple Lys residues.

signal. To investigate whether the interaction between Smo and Herc4 is regulated by Smo phosphorylation, we co-expressed HA-Herc4 with Myc-Smo, Myc-SmoSA, or Myc-SmoSD in S2 cells, respectively. In co-IP experiments, S2 cells transfected with the indicated plasmids and treated with the proteasome/lysosome inhibitors MG132/NH₄Cl, we found that Herc4 appears to interact equally well with Myc-Smo, Myc-SmoSA, and Myc-SmoSD (Figure 8C), suggesting that the PKA-primed Smo phosphorylation does not affect Smo/Herc4 interaction. Considering Hh treatment inhibited the interaction between Smo and Herc4, we

speculate that Hh may regulate the Smo and Herc4 interaction in a manner independent of PKA-primed phosphorylation.

Our data suggested that Hh prevents Herc4-mediated Smo ubiquitination (Figure 8B). This let us investigate the relationship between Smo phosphorylation and Herc4-mediated ubiquitination. As shown in Figure 8D, we found that Herc4 increased the ubiquitin level of Smo, SmoSA, but did not affect the ubiquitin level of SmoSD in S2 cells. Consistently, Herc4 apparently downregulated the protein levels of Smo and SmoSA, but had no effect on the protein level of SmoSD (Figure 8E).

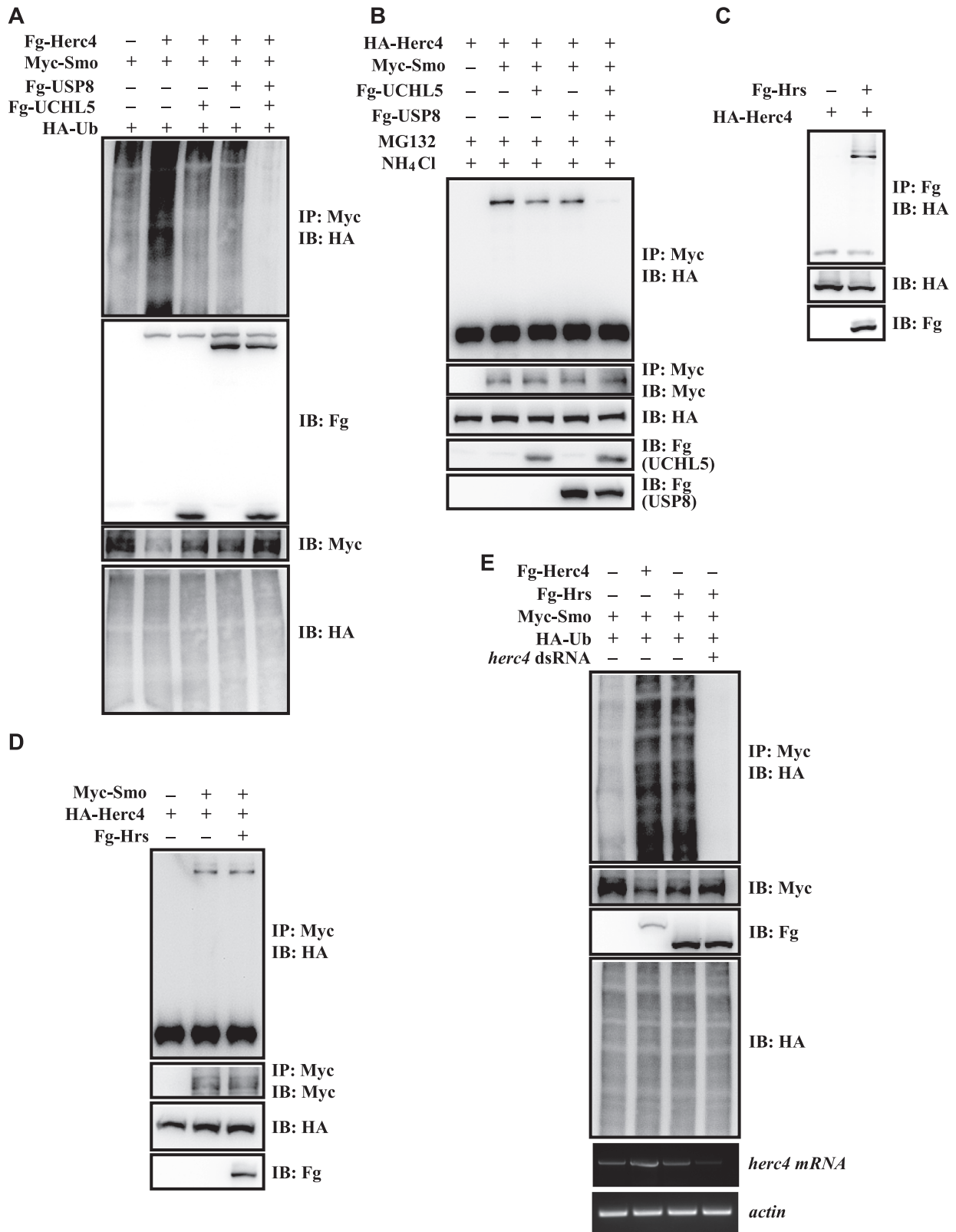


Figure 7 Herc4 interacts with Usp8, UCHL5 and Hrs to regulate Smo ubiquitination. **(A)** Fg-USP8 and Fg-UCHL5 decreased ubiquitination of Smo mediated by Herc4. **(B)** Fg-USP8 and Fg-UCHL5 attenuated the interaction between Herc4 and Smo. **(C)** Fg-Hrs interacted with HA-Herc4 in S2 cells. **(D)** Fg-Hrs did not affect the interaction between HA-Herc4 and Myc-Smo. **(E)** Knockdown of *herc4* decreased the ubiquitin level of Smo mediated by Hrs. *herc4*-dsRNA could effectively knockdown *herc4* mRNA level in S2 cells (bottom two panels).

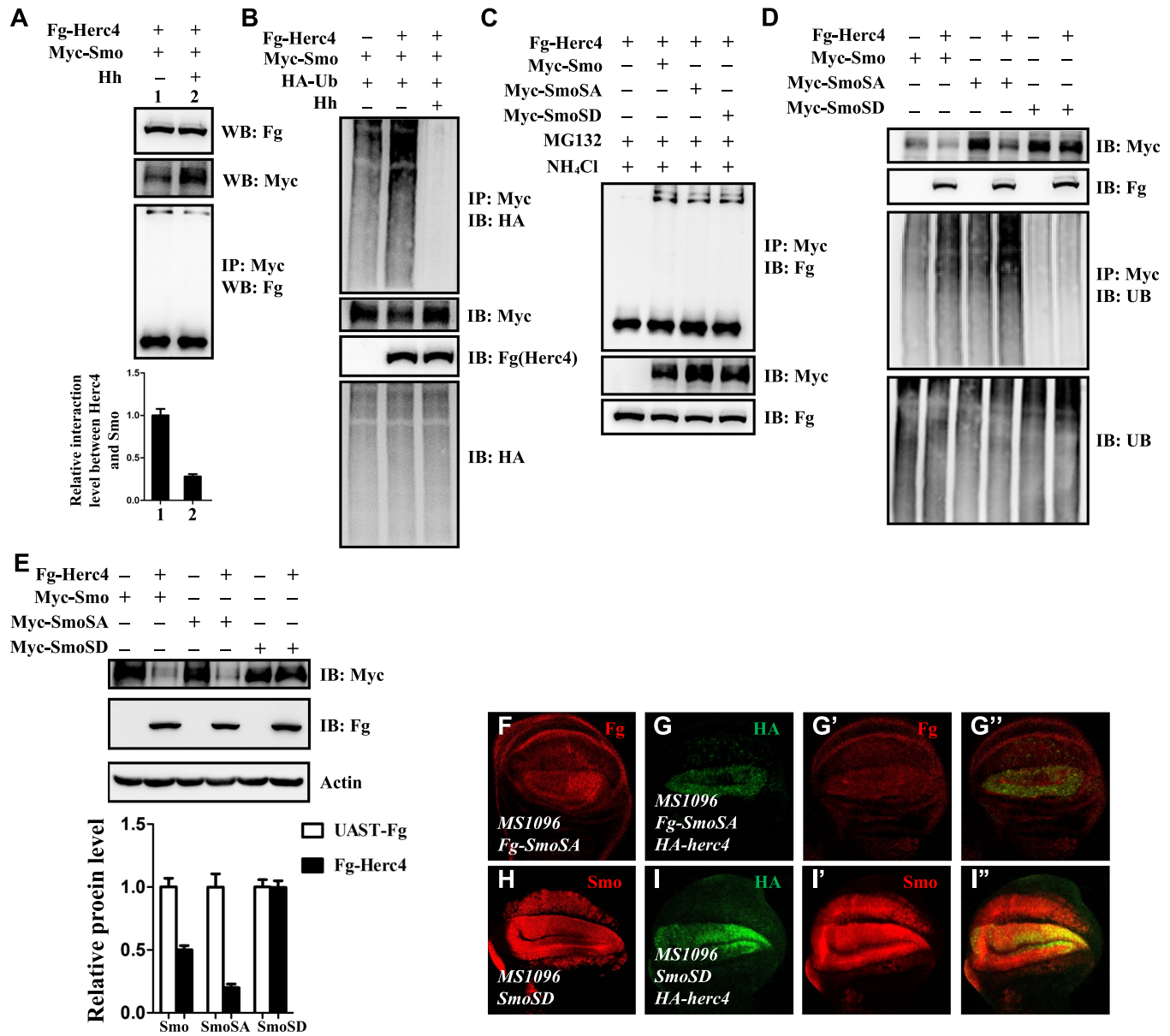


Figure 8 Hh inhibits Herc4-mediated Smo ubiquitination. (A) Hh treatment inhibited the interaction of Herc4 and Smo. (B) Hh decreased Smo ubiquitination mediated by Herc4. (C) S2 cells were transfected with indicated plasmids and treated with the proteasome/lysosome inhibitors MG132 and NH₄Cl. Fg-Herc4 interacts equally with Myc-Smo, Myc-SmoSA, and Myc-SmoSD. (D) From cell-based ubiquitin assay, Herc4 upregulated the ubiquitination level of Smo and SmoSA, but did not affect the ubiquitination level of SmoSD. (E) Overexpression of Herc4 in S2 cells apparently decreased the protein level of Smo and SmoSA, but did not affect SmoSD protein level. (F–G’’) Overexpression of Herc4 in wing discs with *MS1096-gal4* decrease the expression level of Fg-SmoSA (compare G’ with F). (H–I’’) Overexpression of Herc4 in wing discs with *MS1096-gal4* has no effect on the protein level of SmoSD (compare I’ with H).

To further verify this data *in vivo*, we co-expressed Herc4 with SmoSA or SmoSD in wing discs using *MS1096-gal4* driver. As shown in Figure 8F–I’’, overexpression of Herc4 reduced Fg-SmoSA protein level (Figure 8F–G’), but had no apparent effect on SmoSD protein level (Figure 8H–I’).

Taken together, our data suggest that PKA-primed Smo phosphorylation does not affect Herc4 and Smo interaction, instead it affects Smo ubiquitination and protein levels regulated by Herc4.

Discussion

The regulation of Smo intracellular trafficking contributes to transducing the graded Hh signal (Wilson and Chuang, 2010). In the absence of Hh, Smo undergoes ubiquitination in the cytoplasm and fails to accumulate on the cell membrane, culminating in turning off Hh pathway and target genes expression. In the presence of Hh, Ptc relieves its inhibitory effect on Smo by binding Hh, resulting in Smo cell membrane accumulation and Hh pathway activation (Jia et al., 2004; Li et al., 2012; Jiang

and Jia, 2015). The ubiquitination of Smo and its localization are tightly linked. In this study, we identified the HECT domain protein Herc4 as a negative regulator of Hh signalling through genetic screening. We further provided both genetic and biochemical evidences that Herc4 binds and ubiquitinates Smo to modulate its stability. We also demonstrated that Herc4-mediated Smo ubiquitination and protein degradation is inhibited by Hh. Our study that unveils a novel E3 ligase of Smo will deepen our understanding of Hh signalling regulation.

Herc4 belongs to the HECT family of ubiquitin ligases featured by the presence of a HECT domain and one or more RCC1-like domains. We found that both HECT and RCC1-like domains of Herc4 could form a complex with Smo. In addition, both N- and C-terminus of Smo interact with Herc4. Multiple sites interaction may facilitate the flexibility of Smo level regulation. Compared with SmoN, SmoC, especially the SAID domain, supplies both the binding and the ubiquitination sites for mediating Smo degradation by Herc4.

Previous study showed that Hrs binds Smo to promote Smo ubiquitination and degradation (Fan et al., 2013). In our study, we found that Hrs could bind Smo, Herc4 and Hrs promoted Smo ubiquitination via Herc4. USP8 and UCHL5 deubiquitinases have been characterized to downregulate Smo ubiquitination (Li et al., 2012; Xia et al., 2012; Zhou et al., 2018). We found that Herc4 promoted Smo ubiquitination, but it was attenuated by USP8 and UCHL5 (Figure 7A). Multiple deubiquitinases involved in regulation of Smo ubiquitination may facilitate Smo efficient recycling, especially in the presence of Hh signalling.

A recent study suggested that Smo is ubiquitylated at multiple lysine residues in the SAID domain (Li et al., 2012). Consistently, our data showed that Herc4 mediated Smo ubiquitination at multiple lysine residues. Different lysine residues ubiquitination and different ubiquitin-types may guide the Smo protein into the proteasome- and lysosome-mediated degradation. Many studies have verified that Smo undergoes both the proteasome- and lysosome-mediated degradation (Molnar et al., 2011; Li et al., 2012; Xia et al., 2012). Our data showed that Herc4 mediated Smo degradation by proteasome and lysosome, but mainly by proteasome.

Hh blocks Smo ubiquitination, which is vital for Smo activity. What is the underlying mechanism? In this study, we found that Hh regulates Smo stability by Herc4 in two ways. On the one hand, Hh inhibited Herc4–Smo association (Figure 8A), interestingly, it is not dependent on the PKA-primed phosphorylation. Based on our published paper (Zhou et al., 2018) and result shown in Figure 7B, it may be caused by the competing binding of UCHL5. On the other hand, Hh inhibited Herc4-mediated Smo ubiquitination, this time, it is dependent on the PKA-primed phosphorylation (Figure 8B and D). It is possible that PKA-primed phosphorylation may cause conformational change of Smo, which somehow hinder the efficient ubiquitination of nearby Smo Ks sites. Together, the results suggest that Hh blocks Smo ubiquitination by Herc4 through inhibiting Herc4–Smo interaction and stimulating PKA-primed phosphorylation.

Materials and methods

Constructs, mutants, and transgenes

The constructs for expression in S2 cells, including Myc-Ci, HA-Fu, HA-Ub, Fg-Smo, Myc-Smo, Myc-SmoN, Myc-SmoC, Myc-SmoSAID, Myc-SmoCΔSAID, and Myc-SmoΔSAID constructs, have been previously described (Doerks et al., 2002; Zhao et al., 2007; Zhou et al., 2015). To construct Fg-Herc4 and HA-Herc4, we amplified the corresponding cDNA fragments using DNA polymerase (Vazyme), and then cloned them into pUAST-3×Fg or pUAST-3×HA vectors. HA-Herc4^{C1030A} was generated using PCR-based site-directed mutagenesis at the background of HA-Herc4. The HA-tagged Herc4-RCC and Herc4-HECT were constructed by inserting the corresponding coding sequences into the pUAST-3×HA vector. The RNAi lines for the screen were obtained from the Vienna *Drosophila* RNAi Center, including the *herc4* RNAi line, v37220, Bloomington *Drosophila* Stock Center (BDSC), and National Institute of Genetics. The mutant of *herc4*, *herc4*^{G5486}, was obtained from BDSC. *MS1096*, *Smo*^{-PKA}, *ptc-lacZ*, *apG4*, and UAS-GFP have been described (Flybase) (Zhang et al., 2006). The HA-Herc4 and HA-Herc4^{C1030A} transgenic flies were generated by injection of constructs into *Drosophila* embryos according to the methods described previously. The parental strain for all germline transformations is w¹¹¹⁸. All stocks used in this study were maintained and raised under standard conditions.

Immunostaining of wing discs

Immunostaining and *in situ* hybridization of imaginal discs were performed with standard protocols (Zhou et al., 2015). Images were captured with FV10-ASW 2.0 Olympus confocal microscope. Primary antibodies were used at the following dilutions: mouse anti-Smo (1:100; DSHB); mouse anti-β Gal (1:500; Sigma); mouse anti-En (1:50; DSHB); mouse anti-Ptc (1:200; DSHB); rabbit anti-Fg (1:200; Thermo); mouse anti-HA (F7) (1:200; Santa Cruz); anti-ubiquitin (P4D1) (1:50, Santa Cruz); mouse anti-Myc (9E10) (1:200; Santa Cruz); and DAPI (1:1000; Santa Cruz). Secondary antibodies used in this study were bought from Jackson ImmunoResearch, and were diluted at 1:500.

Cell culture, transfection, immunoprecipitation, western blot, cell immunostaining

S2 cells were maintained at 25°C in Schneider's *Drosophila* medium (S9895, Sigma) supplemented with 10% FBS (F0718, Gibco), 100 U/ml penicillin (Life Technologies), and 100 µg/ml streptomycin (Life Technologies). Transfection of S2 cells was performed using calcium phosphate according to the manufacturer's instructions (Invitrogen). For S2 cell immunostaining, 48 h after transfected, cells were harvested and washed with PBS. Cells were fixed in 4% formaldehyde in PBS buffer for 20 min at room temperature. Then, cells were treated with PBT for 20 min, washed with PBS for 20 min three times. To mark the nucleus, cells were stained with DAPI for 20 min after secondary antibody incubation. Images of cells were acquired under FV10-

ASW Olympus confocal microscope. The following antibodies were used for immunoprecipitation and western blot: mouse anti-HA (F-7) (1:2500; Santa Cruz); mouse anti-Myc (9E10) (1:2500; Santa Cruz); mouse anti-Fg (M2) (1:5000; Sigma); LysoTracterRed (1:500; Sigma); MitoTracterRed (1:500; Sigma); ERTracterGreen (1:500; Sigma); mouse anti-Actin (1:5000; AbClonal Technology); goat anti-mouse HRP (1:10000; Abmax). The blots were visualized using a chemiluminescent detection kit (Millipore).

RNA interference

To knock down genes in S2 cells, the double-stranded RNA (dsRNA) was generated by MEGAscript High Yield Transcription Kit (Ambion) according to the manufacturer's instructions. The primer sequences of *herc4* dsRNA are as follows: *herc4*-RNAi-Fw, 5'-GAATTAATACGACTCACTATAGGGAGAGGGCGGCACCTTGCTAC-3', *herc4*-RNAi-Bw, 5'-GAATTAATACGACTCACTATAGGGAGACTGGGAC TGAATGACGAAAC-3' and *herc4*-RNAi-5'UTR-Fw, 5'-GAATTAATACG ACTCACTATAGGGAGATCCCAACCAGTTGGCATCGCC-3', *herc4*-RNAi-5' UTR-Bw, 5'-GAATTAATACGACTCACTTAGGGAGAATTCTTTGTGCCAC TTGCTCC-3' were generated by PCR and used for generating dsRNA. dsRNA targeting the GFP full-length coding sequence was used as a control. The dsRNA-mediated gene knockdown was performed as previously described, S2 cells were cultured in serum-free medium containing dsRNA for 12 h at 25°C before transfection with DNA constructs. Then the culture medium was changed to serum medium for transfection and additional culturing for 48 h (Lawrence Lum et al., 2003; Li et al., 2012).

Protein stability assays

S2 cells were plated in 10-cm dishes and transfected with plasmids after 18–24 h. After another 24 h, the cells were split and transferred into 6-well cell culture plates at equivalent densities. Cells were treated with 20 µg/ml CHX (Calbiochem) for the indicated times before harvesting. After western blots, the band intensity was measured by Image J.

Ubiquitination assays

S2 cells were transiently transfected with the indicated combinations of expression vectors. Four hours before cells harvesting, MG132 (Calbiochem) was added to the media at a final concentration of 10 µM. The ubiquitination assays were then carried out based on the previously described protocol. Briefly, cells were lysed with denaturing buffer (1% SDS, 50 mM Tris-base, pH 7.5, 0.5 mM EDTA, and 1 mM DTT) and incubated at 100°C for 5 min. The lysates were then diluted 10-fold with regular lysis buffer and subjected to immunoprecipitation and western blot analysis.

Real-time quantitative PCR

For real-time quantitative PCR, tissues or cells were lysed in TRIzol (Invitrogen) for RNA isolation following standard protocols. All of the cytosol fraction and nuclei pellet RNA were extracted and the reverse transcription was carried out using PrimeScriptRT Reagent Kit with gDNA Eraser (TaKaRa). The primer

pairs used were as follows: *herc4*, 5'-CCAGCCGGAGGAGCTGATGG CCGTG-3' (forward) and 5'-GTAAAAAGAGGAGAAAACCTCTC-3' (reverse); *Smo*, 5'-CTCACCGACTCCACACCGAAAAG-3' (forward) and 5'-GGACGTCCCAATCGATGGGAAG-3' (reverse); *actin*, 5'-CGAAGAAGTTGCTGCTCTGGTTGTCG-3' (forward) and 5'-GGACGTCC CACAATCGATGGGAAG-3' (reverse).

Statistical analysis

Imaging data were analysed in the program Image J. The data shown in the figures were representative of three or more independent experiments and were analysed by Student's *t*-test, *P* < 0.05 was considered significant.

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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