An Intracellular Activation of Smoothened that is Independent of Hedgehog Stimulation in Drosophila

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An intracellular activation of Smoothened that is independent of Hedgehog stimulation in Drosophila

Kai Jiang, Yajuan Liu, Jie Zhang and Jianhang Jia*

ABSTRACT

Smoothened (Smo), a GPCR family protein, plays a critical role in the reception and transduction of Hedgehog (Hh) signal. Smo is phosphorylated and activated on the cell surface; however, it is unknown whether Smo can be intracellularly activated. Here, we demonstrate that inactivation of the ESCRT-III causes dramatic accumulation of Smo in the ESCRT-III/MVB compartment, and subsequent activation of Hh signaling. In contrast, inactivation of ESCRTs 0–II induces mild Smo accumulation in the ESCRT-III/MVB compartment. We provide evidence that Kurtz (Krz), the Drosophila β-arrestin2, acts in parallel with the ESCRTs 0–II pathway to sort Smo to the multivesicular bodies and lysosome-mediated degradation. Additionally, upon inactivation of ESCRT-III, all active and inactive forms of Smo are accumulated. Endogenous Smo accumulated upon ESCRT-III inactivation is highly activated, which is induced by phosphorylation but not sumoylation. Taken together, our findings demonstrate a model for intracellular activation of Smo, raising the possibility for tissue overgrowth caused by an excessive amount, rather than mutation of Smo.

KEY WORDS: ESCRT, Hh, Smo, Vps20, Vps32, Signal transduction

INTRODUCTION

Hedgehog (Hh) signaling controls organ development, tissue homeostasis and body patterning. Malfunction of Hh signaling causes birth defects as well as several types of cancer (Briscoe and Thérond, 2013; Jiang and Hui, 2008). In Drosophila, the Hh signal is transduced at the plasma membrane, where the receptor complex Patched–Interference Hh (Ptc–Ihog) and the signal transducer Smoothened (Smo) are located. Hh binding to Ptc–Ihog relieves Ptc-mediated inhibition of Smo, which results in Smo activation and then the activation of the Cubitus interruptus (Ci)/Gli family of zinc finger transcription factors, and ultimately induces the expression of Hh target genes, such as decapentaplegic (dpp), ptc, collier (col) and engrailed (en) (Hooper and Scott, 2005; Jia and Jiang, 2006). Smo is a member of the G protein-coupled receptor (GPCR) family and acts as a key regulator of the pathway for both insects and vertebrates. Abnormal activation of Smo results in basal cell carcinoma (BCC) and medulloblastoma; thus Smo has been an attractive therapeutic target exemplified by recent US Food and Drug Administration (FDA)-approved Smo inhibitors for treating cancer driven by Smo dysregulation (Pak and Segal, 2016).

Studies have shown that Hh induces cell surface accumulation and phosphorylation of Smo by protein kinase A (PKA), casein kinase 1 (CK1), casein kinase 2 (CK2), and G protein-coupled receptor kinase 2 (Gprk2) (Chen and Jiang, 2013; Jiang and Jia, 2015), as well as atypical PKC (aPKC) (Jiang et al., 2014). Differential phosphorylation and gradual conformational change in Smo mediates the transduction of Hh activity gradient (Fan et al., 2012; Zhao et al., 2007). The cell surface accumulation of Smo is also regulated by endocytosis; ubiquitylation promotes Smo endocytosis and de-ubiquitylation by Ubiquitin-specific protease 8 (USP8) inhibits Smo endocytosis, which ultimately promotes its cell surface accumulation (Li et al., 2012; Xia et al., 2012). Membrane accumulation and activation of Smo is further regulated by small ubiquitin-like modifier (SUMO) through sumoylation (Ma et al., 2016; Zhang et al., 2017). These findings indicate that cell surface accumulation promotes the activation of Smo; however, it is unknown whether and how Smo can be activated intracellularly.

Many GPCRs use the intracellular route to activate downstream genes. Studies on GPCRs have demonstrated that activation of receptors, and consequent activation of downstream components, often stimulate the receptor endocytosis (Irannejad and von Zastrow, 2014; Sorkin and von Zastrow, 2009). This process may function as a feedback regulation to prevent excessive ligand-induced activation of downstream effectors (Sorkin and von Zastrow, 2009). In addition, GPCR phosphorylation and their association with β-arrestins play a major role in this process (Irannejad and von Zastrow, 2014). Molecular sorting machineries determine their ultimate trafficking fate, as receptors either recycle back to the cell surface or are targeted for lysosomal degradation. Unlike other GPCRs, Smo is activated and accumulated on the cell surface upon Hh stimulation. It is unknown whether Smo undergoes intracellular signaling along the endocytic pathway.

The endosomal sorting complex required for transport (ESCRT) machinery includes four ESCRT complexes (ESCRT-0, -I, -II, -III) and Vacuolar protein sorting 4 (Vps4), and these machineries mediate the selection and transport of proteins destined for lysosomal degradation (Hurley and Hanson, 2010; Wollert and Hurley, 2010). ESCRT facilitates ubiquitylated protein trafficking from endosomes to lysosomes via multivesicular bodies (MVBs) (Henne et al., 2011; Raiborg and Stenmark, 2009; Williams and Urbé, 2007). Clathrin-dependent endocytosis mediates the activation of epidermal growth factor receptor (EGFR), which is further sorted to the MVB then is trafficked to the lysosome (Goh et al., 2010). The ESCRTs 0–III protein complexes drive MVB cargo selection; however, ESCRT-III does not directly interact with ubiquitin. Many membrane-bound proteins are actively sorted to different places in the cell from the early endosome (Hurley and Hanson, 2010). In Drosophila Smo trafficking, the homologs of the ESCRT members mediate sorting of ubiquitylated Smo to endosomes. Smo accumulation is observed upon the inactivation of the ESCRT proteins, such as the Hepatocyte growth factor-regulated...
tyrosine kinase substrate (Hrs), a protein that acts within ESCRT-0 (Fan et al., 2013; Li et al., 2012), Tumor susceptibility gene 101 (Tsg101) in ESCRT-I (Fan et al., 2013; Li et al., 2012), and Vps36 in ESCRT-II (Yang et al., 2013). However, inactivation of the proteins of the early endosome, late endosome, or the ESCRTs 0–II causes a mild accumulation of Smo, which is thought to contribute to the cell membrane accumulation and activation of Smo. It is unknown whether Smo can be activated in the intracellular compartments, similar to the activation of many other GPCRs.

In this study, we found that inactivation of the ESCRT-III core subunits, Vps32 (also known as Shrub in Drosophila, Snf7 in yeast, and CHMP4 in mammal) and Vps20 (CHMP6 in mammals), caused a dramatic increase in Smo. Surprisingly, inactivation of either Vps32 or Vps20 induced ectopic Hh target gene expression at higher levels compared to that induced by the inactivation of proteins in the ESCRTs 0–II. Furthermore, we found that Smo was activated in ESCRT-III-containing compartments, which did not rely on Hh stimulation and was not inhibited by Ptc, indicating that Smo activation can occur in an Hh- and Ptc-independent manner. Moreover, Smo ubiquitylation and sumoylation were not changed in ESCRT-III-containing compartments. To explore the mechanism for intracellular Smo accumulation, we identified a Krz-mediated pathway, operating in parallel to endocytosis, that directs Smo to the ESCRT-III complex and the MVB, which accounts for the high accumulation and activation of Smo. These findings indicate that Smo can be intracellularly activated, and that activated Smo at a certain threshold may result in high levels of Hh signaling activity.

RESULTS

Inactivation of ESCRT-III results in a high level of Smo and activates Hh target gene expression

Previous studies have shown that Smo undergoes lysosomal degradation that is triggered by ubiquitin-mediated endocytosis (Fan et al., 2013; Li et al., 2012; Xia et al., 2012). However, it has been puzzling that, in cultured S2 cells, the majority of Smo is present in large puncta that do not colocalize with ubiquitin (Fig. S1A). In Drosophila wing discs, the developing wing, posterior (P) compartment cells express and secrete Hh that activates adjacent anterior (A) compartment target gene expression, which is mediated by the Ci transcription factor. The low level of Smo in A-compartment cells away from the A/P boundary indicates a rapid protein degradation (Fig. 1A). In wing discs treated with bafilomycin A1 (BFA1) to selectively inhibit vacuolar ATPases that mediate the delivery of internalized proteins from MVB to lysosome, Smo was present in puncta in A- and P-compartment cells (Fig. S1C, compare to Fig. S1B), suggesting that Smo is indeed trafficked from the MVB to the lysosome for degradation. It is possible that the large puncta of Smo observed in S2 cells are located in larger intracellular compartments, such as the MVB.

To better understand the intracellular trafficking of Smo, we collected RNAi lines from different sources to target ESCRT protein expression. We found that knockdown of Vps32 by RNAi resulted in a high level of accumulation of Smo and Ci (Fig. S2A). The level of Ptc protein was also increased in A-compartment cells, indicating activation of the Hh pathway (Fig. S2B). To more precisely examine the accumulation of Smo in wing discs, we used either P35, a cell death inhibitor, or the inducible Gal80° system to prevent cell death induced by Vps32 RNAi. We found that Smo showed high levels of accumulation under both conditions (Fig. 1B, C, gray panel). We also found that the Smo accumulation caused by Vps32 RNAi in large part did not colocalize with Hrs (marks the ESCRT-0 complex) or Rab7 (marks the late endosome) (Fig. 1D; Fig. S2C), but mostly colocalized with lysosome-associated membrane protein 1 (LAMP1) (Fig. 1E), which labels the MVB and lysosome (Pulipparacharuvil et al., 2005; Williams and Urbé, 2007). The expression of LAMP1–GFP alone did not cause any phenotype in vivo (Pulipparacharuvil et al., 2005), and did not change Smo accumulation (Fig. S2D). GFP-tagged Vps32 (Vps32–GFP) has shown to be a dominant negative (Sweeney et al., 2006). We found that Vps32–GFP colocalized with Smo, and induced Smo and Ci accumulation in the wing disc, likely by blocking the function of endogenous Vps32 (Fig. S2E,F). These data suggest that the inactivation of Vps32 causes a dramatic accumulation of Smo, which is likely localized in the MVB.

To examine whether the inactivation of Vps32 regulates the activity of Smo, we first used a sensitized genetic background by expressing the partial dominant-negative SmoDN that we previously described as Smo–PKA12. In this protein, two PKA phosphorylation sites are mutated to avoid phosphorylation (Jia et al., 2004). Expressing SmoDN through the wing-specific C765-Gal4, a weaker Gal4 line than MS1096-Gal4, resulted in a reproducible phenotype of partial fusion between vein 3 and vein 4, indicative of partial loss of Hh signaling activity (Fig. 1H, compare to WT wing in Fig. 1G). We found that Vps32 RNAi reversed the activity of SmoDN, resulting in a rescue of the vein 3–vein 4 fusion phenotype (Fig. S2I). In comparison, Vps32 RNAi driven by C765-Gal4 produced a mild phenotype in the adult wing (Fig. S2J). These data indicate that inactivation of Vps32 regulates the activity of Smo. We further examined Smo accumulation in Vps32 mutant cells and found that Smo showed a dramatic accumulation in Vps32 mutant cells (Fig. 1F). Surprisingly, mutating Vps32 induced expression of En, a marker for the peak activation of Hh signaling (Fig. 1G). We next investigated cultured S2 cells and found that the levels of Smo were increased upon both Vps32 RNAi and Hh treatment, and that the accumulation of Smo reached a peak level when Vps32 RNAi and Hh treatment were combined (Fig. 1H). Consistent with this, Vps32 RNAi significantly increased the ptc-lucerase (ptc-luc) reporter activity, which was further increased upon Hh treatment (Fig. 1I). In these experiments, very high RNAi efficiency was achieved and monitored by quantitative real-time PCR (qRT-PCR) (Fig. 1J). In a cell-based assay to examine Smo cell surface accumulation, Hh treatment consistently increased the accumulation of Smo on the cell surface (Jia et al., 2004) (Fig. 1K); however, the inactivation of Vps32 significantly increased Smo accumulation inside the cell but not on the cell surface (Fig. 1K). These data suggest that Smo can be intracellularly activated during a malfunction of ESCRT-III.

To further examine the ESCRT-III-mediated regulation of Smo accumulation and activation, we used a mutant of Vps20, which is another critical protein in the ESCRT-III complex. We found a high accumulation of Smo in cells with mutated Vps20 (Fig. 2A). Consistent with the finding that mutating Vps32 induced Hh target gene expression (Fig. 1G), mutation of Vps20 induced ectopic expression of dpp-lacZ and ptc-lacZ, indicating that Smo activates Hh target gene expression in cell autonomous manner (Fig. 2B,C; Fig. S3 for higher magnification images). In cultured cells, we found that knockdown of Vps20 by RNAi significantly increased the ptc-luc activity (Fig. 1I); however, it did not increase the amount of cell surface-localized Smo (Fig. 1K). These data confirmed the activation of Smo upon ESCRT-III loss of function. These findings were very interesting because inactivation of the ESCRT-0, ESCRT-I and ESCRT-II increases the cell surface accumulation of Smo but does not significantly increase Hh target gene expression.
Fig. 1. See next page for legend.
Smo is phosphorylated and activated in ESCRT-III-containing compartments

To determine the active/inactive status of Smo in the intracellular compartment, we first examined the ability of Ptc to inhibit Smo activation. We found that overexpression of Ptc inhibited Smo accumulation and decreased the level of Ci in wild-type cells of the wing disc but not in Vps20-depleted cells (Fig. 2D). We also examined the levels of endogenous Ptc protein and found that Ptc accumulated upon Vps20 mutation (Fig. 2E); however, the accumulated Ptc, similar to exogenously expressed Ptc protein, did not inhibit Smo (Fig. 2A). These data suggest that the accumulation of Smo in the ESCRT-III-containing/MVB compartment is not inhibited by Ptc. To further examine the localization of Smo, we immunostained the wing disc for Vps4 that labels protein localization close to the ESCRT-III/MVB (Rodahl et al., 2009). We found that Smo colocalized with Vps4 in cells mutating Vps20 (Fig. 2F). Taken together with the finding that RNAi interference of Vps32 led to the accumulation of Smo (in puncta marked by LAMP1), our data suggest that the inactivation of ESCRT-III causes Smo accumulation in the MVBs.

Inactivation of ESCRT-III results in an accumulation of different forms of Smo

The unchanged levels of Smo sumoylation and ubiquitylation upon Vps32 inactivation led us to examine the stability of the different forms of Smo to determine which form of Smo accumulates in the ESCRT-III-containing compartment. We carried out a protein stability analysis by transfecting S2 cells with Myc–SmoWT, Myc–SmoPKA123 (the phosphorylation-deficient, inactive form of Smo) and Myc–SmoSD123, and then monitoring Smo levels upon the inactivation of Vps32. We then performed western blot analysis to examine the stability of the different forms of Smo and found that knockdown of Vps32 by RNAi did not change the levels of ubiquitylation for either wild-type Smo (SmoWT) or the phosphorylation-mimetic active form of Smo (SmoSD123) (Fig. 3H). These findings suggest that intracellular Smo activation does not require sumoylation and is not mediated by deubiquitylation, pointing to a different mechanism from that utilized for cell membrane Smo activation.
Fig. 2. Vps20 loss-of-function induces high levels of Smo accumulation and Hh signaling activation. (A) A wing disc carrying vps20 mutant clones was immunostained for Smo, Ci and GFP. Clones are marked by the lack of GFP expression. Arrows indicate high accumulation of Smo and Ci. (B,C) Wing discs carrying vps20 mutant clones were immunostained for GFP, dpp-lacZ (DppZ) or ptc-lacZ (PtcZ). The arrows identify the ectopic expression of dpp-lacZ and ptc-lacZ. (D) A wing disc carrying vps20 mutant clones and expressing UAS-HA-Ptc driven by MS1096-Gal4 was immunostained for Smo, Ci and GFP. The arrows show a high accumulation of Smo and Ci not inhibited by Ptc expression in vps20 mutant cells. The arrowheads identify the inhibition of Smo and Ci accumulation in vps20 wild-type cells. (E) A wing disc carrying vps20 mutant clones was immunostained for Ptc and GFP. The arrow indicates a high accumulation of Ptc protein. (F) High-magnification of a wing disc carrying vps20 mutant clones and immunostained for Smo, Vps4, and GFP. Arrows indicate the colocalization of Smo with Vps4.
Fig. 3. Smo is phosphorylated and activated by Vps32RNAi in wing disc. (A–C) Wing discs from flies expressing UAS-Vps32RNAi and UAS-HhRNAi alone or together driven by MS1096-Gal4 were stained for Smo, ptc-lacZ (PtcZ) and Ci. A control experiment is shown in C, with arrows indicating the blocked accumulation of Smo (arrow in gray image), inhibited expression of ptc-lacZ (arrow in green image) and inhibited activation of Ci (arrow in red image). Of note, co-expressing Vps32RNAi with HhRNAi caused a very similar phenotype to that seen upon expressing Vps32RNAi alone. (D,E) Higher magnification images of wing discs from flies expressing Myc–SmoWT alone or together with Vps32RNAi. Discs were immunostained with the anti-Myc, anti-Ci and anti-SmoP antibodies. Of note, the anti-SmoP antibody can detect phosphorylation of the overexpressed Smo. (F) S2 cells were transfected with Myc–SmoWT followed by the treatment with Vps32dsRNA or HhN-conditioned medium. Cell extracts were immunoprecipitated (IP) with the anti-Myc antibody and analyzed by western blotting (WB) using either the anti-SmoP antibody to detect Smo phosphorylation or the anti-Myc antibody to monitor the expression of Myc–SmoWT. Western blots for GFP served as a transfection and loading control. Smo was normalized by previously described methods (see Materials and Methods). (G) A cell-based assay to detect the levels of Smo sumoylation. S2 cells were co-transfected with Myc–SmoWT, HA–SUMO and Flag–Ubc9, followed by the treatment with Vps32dsRNA, HhN-conditioned medium or control medium. Cell extracts were immunoprecipitated with the anti-Myc antibody and analyzed by western blotting using the anti-HA antibody to examine the Smo-bound SUMO, or with the anti-Myc antibody to monitor the levels of Smo. Western blots for GFP served as a transfection and loading control. Smo was normalized by the previously described methods (see Materials and Methods). (H) To examine Smo ubiquitylation, S2 cells were transfected with the indicated constructs followed by treatment with Vps32dsRNA. Cell lysates were immunoprecipitated with the anti-Myc antibody and analyzed by western blotting using the anti-HA antibody to examine the Smo-bound ubiquitin, or with the anti-Myc antibody to monitor the levels of Smo. Western blots for GFP served as a transfection and loading control. Myc–SmoWT and Myc–SmoSD123 were normalized separately.
therefore performed a more precise analysis to examine the stability of the Smo protein at different time points after the treatment by using the protein synthesis inhibitor cycloheximide (CHX). We found that the half-lives of SmoWT and SmoSD123 were increased upon Vps32 RNAi (Fig. 4C,D, middle panel), compared to the half-lives of these forms of Smo in cells without Vps32 knockdown (Fig. 4C,D, left panel), which was further demonstrated by quantification analyses (Fig. 4C,D, right panel). These data suggest that the active form of Smo is sorted through ESCRT-III for lysosome-mediated degradation. It has been shown that ubiquitylation at lysine residues of Smo C-tail promotes, whereas mutating these residues blocks, Smo endocytosis (Li et al., 2012; Xia et al., 2012; Yang et al., 2013). Interestingly, we found that SmoK42R, with all 42 lysine residues in the Smo C-tail mutated to arginine to block Smo ubiquitylation, accumulated in vps20 mutant cells (Fig. 5B), which was similar to

Fig. 4. The stability of Smo variants regulated by the inactivation of Vps32. (A,B) S2 cells were transfected with the indicated Smo constructs followed by the treatment with Vps32 dsRNA. Cell extracts were immunoprecipitated with the anti-Myc antibody and analyzed by western blotting with the anti-Myc antibody to examine the stability of Smo. For a better comparison, images from shorter and longer exposure times are shown. Western blots for GFP served as a transfection and loading control. (C–E) Smo protein stability assays. S2 cells were transfected with Myc–SmoWT, Myc–SmoSD123 or Myc–SmoK42R, treated with or without Vps32 dsRNA, and incubated with CHX for the indicated time periods. Cell extracts were immunoprecipitated with the anti-Myc antibody and analyzed by western blotting with the anti-Myc antibody to examine the levels of Smo protein. The quantification of Smo levels at different time points are shown in the right panel. Signal density at t=0 was defined as 100%. Data were from three independent experiments for each set. *P<0.05, †P<0.01, ‡P<0.001 versus Smo expression without Vps32 dsRNA (Student’s t-test).
Fig. 5. The signaling activity of different forms of Smo in vivo.

(A,B) Wing discs carrying vps20 mutant clones and expressing Myc–SmoWT or Myc–SmoK42R driven by MS1096-Gal4 were immunostained for GFP to label the clones, and for Myc to examine the levels of Smo inside and outside vps20 clones. The arrows indicate the elevated levels of both SmoWT and SmoK42R in cells with mutated vps20. (C,D) Wing discs carrying smo mutant clones immunostained for Ptc, En, Ci and GFP. Arrows in the red panel indicate the lack of Ptc or En expression upon smo mutation. Arrows in the green panel indicate the clones recognized by the lack of GFP expression.

(E–H) Wing discs containing smo mutant clones and expressing different forms of Smo at the same attP locus (VK5 75B1) driven by the tubulin-α promoter were immunostained for Ptc, En, Ci and GFP. Arrows in the red panel indicate the rescue of Ptc or En expression by the exogenously expressed Smo. Arrows in the green panel show smo mutant clones marked by the lack of GFP expression. Yellow dashed lines indicate the A/P boundary defined by Ci staining.
the observed accumulation of Smo WT in these cells (Fig. 5A). Both Smo WT and Smo K42R were stabilized by the treatment of BFA1 in cultured S2 cells (data not shown). These data suggest that Smo K42R is sorted into the ESCRT-III-containing/MVB compartment through a different route rather than through the endocytosis pathway. In support of this hypothesis, the half-life of Smo K42R was increased by knockdown of Vps32 in the cell-based assay that focused on Smo stability (Fig. 4E). To examine the in vivo activity of Smo K42R, we used a tubulin-α promoter, which gives an expression level that is similar to the endogenous Smo expression level (Jia et al., 2010), and found that Smo K42R rescued ptc and en expression in smo mutant cells (Fig. 5E,F, compare to 5C and 5D, respectively). The activity of Smo K42R may have two components: one from Smo accumulation on the membrane due to the blockade of endocytosis, and the other from the accumulation in the ESCRT-III-containing/MVB compartment.

Many membrane-bound proteins contain a KKDE motif that interacts with COP-I coats, which mediate target protein delivery to the endoplasmic reticulum. Fusion of the KKDE motif at the C-terminus of Smo removed Smo from the cell surface and significantly reduces the activity level of the different forms of Smo (Zhu et al., 2003). To further assess the role of the intracellularly localized Smo in transducing an Hh signal, we generated SmoKKDE, which is consistent with previous findings (Fan et al., 2013; Henne et al., 2011). We found that RNAi-mediated knockdown of Smo, undergoes trafficking/degradation through other mechanisms.

**Krz acts in parallel to the endocytosis pathway to sort Smo to ESCRT-III**

Hrs and Signal transducing adaptor molecule (Stam) constitute the ESCRT-0 complex responsible for the initial selection of ubiquitylated proteins that are ultimately degraded in lysosomes (Henne et al., 2011). We found that RNAi-mediated knockdown of Hrs caused a mild increase in Smo accumulation (Fig. 6C, compared to 6A), which is consistent with previous findings (Fan et al., 2013; Li et al., 2012). We also found a mild accumulation of Smo in hrs mutant cells (Fig. S6A), and that the double mutation of hrs and stem did not increase the accumulation of Smo (Fig. S6B). Doubly mutating hrs and stem was thought to completely block Smo endocytosis mediated by ESCRT-0. Our findings raise the possibility that Smo, especially the non-ubiquitylated forms of Smo, undergoes trafficking/degradation through other mechanisms.

Previous studies showed that Kurtz (Krz), the Drosophila non-visual arrestin, downregulated Smo accumulation by promoting Smo degradation in a ubiquitin- and Gprk2-independent manner (Li et al., 2012; Molnar et al., 2011). Overexpression of Krz prevented Smo accumulation in wing discs but krz mutation did not (Fig. 6E) (Li et al., 2012; Zhang et al., 2017). Similarly, we found that knockdown of Krz by RNAi did not result in any change in Smo accumulation (Fig. 6B, compared to 6A). However, Smo accumulation was markedly increased when Krz RNAi and Hrs RNAi were combined (Fig. 6D, compared to 6B and 6C). In addition, knockdown of Hrs by RNAi in krz mutant cells resulted in the accumulation of Smo to a higher level than in krz wild-type cells (Fig. 6F). It has been shown that Vps32 acts as a key modulator in ligand-independent Notch signaling mediated by Krz and ESCRT-III (Hori et al., 2011). Our findings led to the hypothesis that Krz and Hrs act in parallel pathways to regulate Smo trafficking. To further test this hypothesis, we examined the ability of Krz to inhibit Smo accumulation under a variety of conditions. We found that, consistent with previous findings, Krz overexpression completely blocked Smo accumulation (Fig. 6G). Hrs RNAi caused Smo accumulation in both Krz-expressing cells (Fig. 6H) and wild-type cells (Fig. 6HJ). Furthermore, overexpression of Krz inhibited Smo accumulation in wild-type cells but not in hrs mutant cells (Fig. 6K). These results indicate that Hrs and Krz act in parallel to regulate Smo.

To determine how ESCRT-I regulates Smo, we examined the effect of Vps28 loss of function in the ESCRT-I complex. We found that mutating vps28 induced a moderate accumulation of Smo, similar to that caused by hrs mutation (Fig. S6C and A, respectively). The accumulation of Smo in vps28 mutant cells was not adequate to induce Ci accumulation in the wing disc (Fig. S6C). These data indicate that the same forms of Smo are trafficked from ESCRT-0- to ESCRT-I-containing compartments. Our finding that both RNAi and mutation of Vps32 caused a striking accumulation of Smo in the ESCRT-III-containing compartment (Figs 1 and 2) suggests that Smo may sort to the ESCRT-III through an ESCRT-0-independent pathway. In support of this hypothesis, we found that Krz overexpression did not inhibit the Smo accumulation that was induced by Vps32 RNAi (Fig. 6J). The Vps4 complex acts downstream of ESCRT-III to mediate protein degradation through lysosomes. Similar to what was seen with Vps32 RNAi (Fig. 3A; Fig. S2A), Smo accumulation was observed when a dominant-negative Vps4DN was expressed in wing disc (Fig. S6D). Taken together, our data suggest that the parallel pathways mediated by Krz and Hrs may merge at ESCRT-III to direct additional trafficking and lysosomal degradation.

Autophagy is an evolutionarily conserved, highly regulated cellular process. Autophagosomes can potentially fuse with lysosomes, or with early and late endosomes; however, Vps34 and Beclin 1 play selective roles in the autophagy pathway (Funderburk et al., 2010; Raiborg and Stenmark, 2009). To examine whether Smo is regulated by the autophagy, we examined the accumulation of Smo in both wing disc and cultured S2 cells when autophagy gene expression was knocked down by RNAi. We found that knockdown of either Vps34 or Beclin 1 by RNAi did not cause any changes in Smo accumulation or ptc-lacZ expression in wing disc, although both Vps34 and Beclin 1 RNAi induced phenotypes in the adult wing (Fig. S5A,B, data not shown). Consistent with this, inactivation of other autophagy genes, such as Vps15, Atg8a, Atg8b and Atg7, did not cause any changes in Smo accumulation and Hh target gene expression (data not shown). The phenotypes are unlikely to be due to off-target effects because different lines targeting non-overlapping regions produced similar phenotypes. We also used the cell-based assay to examine Smo stability in S2 cells and found that RNAi for Vps15, Beclin1 and Vps34 did not cause any changes in the levels of Smo (Fig. S5C). Taken together, we conclude that the autophagy pathway is not involved in Smo stability control.

There is a possibility for Krz to mediate Smo degradation through the proteasome (Li et al., 2012). The dilemma was to be able to distinguish Krz-mediated Smo sorting to the MVB from that to the
proteasome. We explored the possibility of an interaction of Krz with different forms of Smo that are representative of different compartments. We found that endogenous Krz strongly interacted with transfected Myc–Smo<sup>WT</sup>, indicating that Krz and Smo were physically associated (Fig. 7A, lane 1, top panel). We further found that Krz interacted with Smo<sup>K42R</sup> at a level comparable to Smo<sup>WT</sup>, indicating that mutating the 42 lysine residues in the Smo C-tail did not change the overall association of Krz with Smo (Fig. 7A, lane 2, top panel). However, Krz interacted with Smo<sup>KKDE</sup> and Smo<sup>FD</sup> at significantly higher levels than with Smo<sup>WT</sup>, suggesting that endoplasmic reticulum-localized Smo (mediated by the KKDE signal) and endocytosed Smo (promoted by the FDNPVY internalization signal) are favorably subjected to Krz-mediated trafficking (Fig. 7A, lanes 3,4, top panel, and quantification analysis in the right panel). These data suggest that Krz physically interacts with the endocytosed and endoplasmic reticulum-localized Smo to promote Smo degradation through lysosomes.

We found that the endogenous Krz protein was very stable and that its stability was not altered by Vps32 RNAi, MG132 (a potent proteasome inhibitor), BFA1 or Hh treatment, suggesting that the endogenous Krz does not undergo degradation through either the proteasome- or lysosome-mediated pathways (Fig. 7B). We also found that the endogenous Krz protein was stable and was expressed in the wing imaginal disc, with a slightly higher level in the wing pouch, but that no difference was observed between the A-compartment and P-compartment cells (Fig. 7C), suggesting that the expression or stability of Krz is not regulated by Hh. These data indicate that endogenous Krz is likely recycled before targeting Smo into the proteasome or lysosome, similar to what occurs for ubiquitin recycling in the endocytic pathway. The overexpressed Krz was also very stable and completely blocked Smo accumulation in P-compartment and A-compartment cells near the A/P boundary (Fig. 6G); however, Krz overexpression did not alter the expression levels of Ci, Ptc and En (Fig. 7D,E). These data indicate that the Smo internalized by Krz was active. This was further supported by the finding that overexpression of Krz did not block the accumulation of Cos2 and Fu, but rather increased the levels of Cos2 and Fu in P-compartment cells (Fig. S7A,B), which indicates that Krz neither inhibits Smo activity nor blocks Smo protein accumulation.
DISCUSSION
The regulation of Smo activation has been widely studied, and mainly focused on the identification and characterization of Smo mutations, including those mutations shown in earlier studies to drive cancer formation (Taipale et al., 2000; Xie et al., 1998). It is unclear whether the accumulation of wild-type Smo under certain conditions can induce the activation of Hh signaling, and thus over-proliferation of cells. In addition, it is unclear whether and how Smo transduces Hh signaling inside the cell, although it has been demonstrated that accumulation of Smo at the cell surface (in Drosophila) or in cilia (in vertebrates) triggers the activation of Smo. In this study, we found that inactivation of the ESCRT-III complex caused severe accumulation of Smo in the MVB and dramatic activation of Hh signaling. In contrast, inactivation of other intracellular components, such as the ESCRTs 0–II, only induced a mild accumulation of Smo. By carrying out genetic epistasis analysis, and by examining the accumulation of different forms of Smo in different cellular compartments, we found that Krz acted in
In this study, we show that Krz mediates the internalization of Smo through a pathway parallel to the endosome/ESCRT-0-mediated endocytosis. However, there is no effect of krz loss-of-function on Smo accumulation and Hh signaling activity (Fig. 6E) (Li et al., 2012; Molnar et al., 2011). This has been puzzling for years. We provide the following explanations. It is possible that other arrestins compensate for the function of krz when krz is lost. It is also possible that Krz functions in parallel to the endocytosis pathway, allowing Smo to be degraded through other pathways when krz is lost. This latter idea is supported by findings in this study, and also supported by the finding that Krz acts in an ubiquitylation-independent manner to guide Smo to both proteasome- and lysosome-mediated degradation (Li et al., 2012).

The third possibility is that endogenous Krz can only regulate the basal level of Smo trafficking and thus the basal degradation of Smo. Moreover, it is possible that Krz-mediated internalization of Smo does not alter the activity of Smo in the intracellular compartment. This hypothesis may explain, at least in part, why overexpressed Krz completely blocks Smo accumulation without any effect on Hh pathway activity (Fig. 7D,E).

MVB sorting is normally mediated by the ESCRT pathway, in which the MVB cargo selection is determined by the ESCRT-0–III protein complexes (Henne et al., 2011; Hurley and Hanson, 2010). ESCRTs 0–II contain ubiquitin-binding proteins and are responsible for the sequestration of ubiquitylated proteins. Hrs, containing a ubiquitin-interacting motif (UIM), forms a heterodimer with Stam to constitute ESCRT-0 complex. ESCRT-I contains Vps23, also known as tumor susceptibility gene 101 (Tsg101), which possesses a ubiquitin E2 variant (UEV) domain responsible for cargo recognition. ESCRT-II contains Vps36, which carries a GRAM-like ubiquitin-binding motif in EAP45 (GLUE) domain also responsible for cargo recognition. ESCRT-III, which does not bind ubiquitin directly, contains Vps32 and Vps20, assembles on endosomes and further connects with Vps4 for subsequent entrapment of GPCRs within the MVB. The ESCRT-III complex is pivotal to cargo capture into MVB (Wollert and Hurley, 2010). The ESCRT/MVB pathway is involved in the trafficking many proteins, and a recent study has found that ESCRT-III and Krz are involved in ligand-independent Notch signaling (Hori et al., 2011). From the view of Smo trafficking, ubiquitylated Smo can be recognized by ESCRT-0, -I, and -II, and can be clustered into the ESCRT-III-containing compartment after deubiquitylation. ESCRT-III does not bind ubiquitin, raising the possibility that Krz-mediated non-ubiquitylated forms of Smo can be sorted to the ESCRT-III-containing compartment with subsequent lysosomal degradation. We speculate that Smo is activated in the MVB independently of ubiquitylation and sumoylation regulation. In support of this concept, we found that the accumulation of Smo upon inactivation of the ESCRT-III did not alter the ubiquitylation and sumoylation of Smo (Fig. 3F,G). The other supportive finding was that the active forms of Smo underwent lysosome-mediated degradation (Fig. 4D). Many lysine residues in the Smo C-tail can be either sumoylated or ubiquitylated (Li et al., 2012; Ma et al., 2016; Xia et al., 2012; Yang et al., 2013; Zhang et al., 2017). It is likely that Smo sumoylation counteracts ubiquitylation to regulate Smo cell surface activation; however, the intracellular activation of Smo does not require sumoylation or deubiquitylation. The intracellularly accumulated Smo was phosphorylated (Fig. 3D,E), which likely induces dimerization and activation of the protein.

It is interesting that the overexpressed Krz blocked Smo accumulation, but increased Fu and CoS2 accumulation in P-compartment cells (Fig. S7A,B). This indicates that the components parallel with the ESCRTs 0–II pathway to promote Smo degradation through the MVB and lysosome. The Smo that accumulated upon the inactivation of ESCRT-III was highly active, which was likely induced by phosphorylation but not sumoylation. We propose a model for Smo intracellular trafficking regulated by different pathways (Fig. 7F,G). The findings in this study uncovered a novel mechanism for Smo activation in the intracellular compartment, raising the possibility for tissue over-proliferation caused by an excessive level of Smo induced by the malfunction of ESCRT-III.

The signaling of many GPCRs is controlled by endocytosis. There are incidents of endocytic regulation at the plasma membrane, regulation of signaling by different endocytic routes and signaling from endosomes (Sigismund et al., 2012). Models have been proposed for GPCR signaling in the endosomes. These include the sustained GPCR signaling from endosomes as is likely to occur for the thyroid-stimulating hormone GPCR (TSHR), and the acute GPCR signaling from endosomes for the D1 dopamine receptor (DRD1) (Irannejad and von Zastrow, 2014). It is not surprising that malfunction of the endosomal trafficking compartments causes Smo accumulation. It is also not surprising that Krz promotes Smo internalization. What is surprising is that Smo can accumulate in the MVB at such an unexpected level as to induce its phosphorylation and activation. It is also surprising that, independently of Ptc inhibition, the activated Smo induces high levels of Hh target gene expression. These findings suggest a model for GPCR activation in ESCRT-III-containing or MVB compartments.
downstream of Smo are accumulated and likely activated by the overexpression of Krz, and suggests that Krz does not inhibit the activity of Smo, Cos2 and Fu, although Krz blocks Smo accumulation in wing disc. Upon examining the levels of Fu and Cos2 in the intracellular compartment, we found that Fu and Cos2 were not accumulated in cells with mutated vps20 (Fig. S7C,D), suggesting that the accumulation and activation of Smo does not promote the accumulation of Fu and Cos2 in these cells. These data also suggest that Krz likely acts upstream of ESCRT-III to regulate Smo trafficking. Taken together with the finding that the intracellular activation of Smo does not require sumoylation, we speculate that the activation of Smo in the intracellular compartment is mediated in a different way from its activation on the cell surface.

**MATERIALS AND METHODS**

**Constructs, mutants and transgenes**

Generation of the Myc–SmoWT, Myc–SmoKD123, Myc–SmoPKA123 and Smo–CFP constructs was previously described (Jia et al., 2010, 2003). vps32 and vps32D were cloned from Bloomington Stock Center (BSC) (#39635 and #39623, respectively) and vps32 mutant clones shown in this study were generated by using the vps32D-mutant construct combined with FRT42D. To generate survival clones, UAS-P35–Gal4 and wing dorsal-compartment-specific Ptc construct (Xia et al., 2012). The HA–Krz transgenic construct and transgenic line have been described previously (Zhang et al., 2017). The HA–Ptc construct and transgenic line have been described previously (Jiang et al., 2016). All the transgenic lines were generated by using the 75B1 VK5-attP locus to sure Smo protein expression at the same levels without positional effects.

The wing-specific MS1096-Gal4 and wing dorsal-compartment-specific ap-Gal4 have been described in our previous studies (Jia et al., 2010, 2003). vps32O3 and vps32D were obtained from Bloomington Stock Center (BSC) (#39635 and #39623, respectively), and vps32 mutant clones shown in this study were generated by using the vps32O3-mutant construct combined with FRT42D. To generate survival clones, UAS-P35–Gal4 was expressed in wing disc through MS1096-Gal4. The HA–Krz transgenic insertion on the second chromosome and the krz mutant line have been described previously (Mukherjee et al., 2005). krz mutant clones were generated by using the krz mutant combined with FRT82B. vps28 mutant clones were generated with the vps28G–FRT42D stock from BSC (#39634). Genotypes for examining the activity of the Smo transgene in smo3 FRT40–mutant clones shown in this study were generated previously (Zhang et al., 2017). The HA–Ptc construct and transgenic line have been described previously (Jiang et al., 2016). All the transgenic lines were generated by using the 75B1 VK5-attP locus to ensure Smo protein expression at the same levels without positional effects.

**Cell culture, immunoprecipitation, western blotting, luciferase reporter assay, cell surface staining, and statistical analysis**

**Drosophila** S2 cells were cultured as previously described (Jiang et al., 2016). Briefly, S2 cells were transfected with various UAST constructs using the Effectene transfection reagent (Qiagen). At 48 h post transfection, cells were treated with lysis buffer (100 mM NaCl, 50 mM Tris-HCl pH 8.0, 1.5 mM EDTA, 10% glycerol, 1% NP-40, and protease inhibitor tablet; Roche), followed by centrifugation at 17,949 g for 10 min. For each sample, 6×10⁶ cells were harvested and lysed in 450 µl lysis buffer. 50 µl was saved for direct western blots, with 4 µl for each load. The remaining 400 µl was used for an immunoprecipitation assay, generating 30 µl samples. For immunoprecipitation, the cell lysate was incubated with the correct primary antibody for 2 h, and beads of protein A ultralink resin were added (Thermo Scientific). Immunoprecipitated protein samples were resolved by SDS-PAGE and transferred onto PVDF membranes (Millipore) for western blotting, which was performed using the indicated antibodies and the enhanced chemiluminescence (ECL) protocol. To normalize the level of Smo, 50 µM MGI32 and 15 mM NH4Cl was used to block Smo degradation, and samples were normalized for loading (Fan et al., 2012). The use of HbN-conditioned medium has been previously described (Fan et al., 2012). For the ubiquitination assay, S2 cells were transfected with Myc–Smo plus HA–Ub and lysed with denaturing buffer (1% SDS, 50 mM Tris-HCl pH 7.5, 0.5 mM EDTA, and 1 mM DTT) and incubated at 100°C for 5 min. The lysates were then diluted with regular lysis buffer and subjected to immunoprecipitation with the anti-Myc antibody and western blotting with the anti-HA antibody. Experimental protocols for MGI32 (Calbiochem), a proteasome inhibitor, and NLHCl (Sigma-Aldrich), a lysosome inhibitor, to block Smo degradation have been previously described (Li et al., 2012; Xia et al., 2012). For BFA1 treatment, 300 nM BFA1 was added to the medium for 4 h before harvesting the cells. The S2 cell treatment with dsRNA has been described previously (Liu et al., 2007). dsRNA was synthesized against a specific region to knock down the following gene expression: Vps32-1 (nucleotides 7–566); Vps32-2 (nucleotides 559 to 460 after the stop codon in the 3′-UTR); Vps20 (nucleotides 21–530); Vps15 (nucleotides 1941–2531); Vps30 (nucleotides 691–1254); Vps34 (nucleotides 2279–2820), and RNAi efficiency for each RNAi was monitored by qRT-PCR. GFP dsRNA was synthesized against nucleotides 6–606 and was used as the control for most RNAi experiments. GFP RNAi efficiency was determined by western blotting using the anti-GFP antibody to detect the expression levels of co-transfected GFP. For qRT-PCR, total RNA was extracted with Trizol reagent (Invitrogen). cdNA was synthesized using SuperScript III First Strand Synthesis kit (Invitrogen).
according to the manufacturer’s instructions. The quantitative real-time PCR reactions were carried out using SYBR Green PCR master mix reagents (Thermo Scientific) on the ABI StepOnePlus real-time PCR system (Applied Biosystems). Thermal cycling was conducted at 95°C for 30 s, followed by 40 cycles of amplification at 95°C for 5 s, 55°C for 30 s and 72°C for 15 s. The following primers were used: Vps32, 5′-GAGCAACGTAATAGACCCG-3′ and 5′-TCATGTCGTGCACCCTTGTC-3′; Vps20, 5′-GATGTGCCCA-CGAAATCT-3′ and 5′-CGAGACTTCTTGGTGCTT-3′.

The cell-based assay to examine Smo cell surface accumulation was carried out by immunostaining with the anti-SmoN antibody before cell permeabilization. After incubation with the anti-SmoN antibody at room temperature for 30 min, cells were washed with PBS and fixed with 4% formaldehyde in PBS for 20 min. After permeabilization with 1% PBST, cells were then incubated with the anti-Myc antibody for 1 h (unless autofluorescence of CFP was to be measured), appropriate secondary antibody for 1 h, washed three times with PBST after each incubation, and finally mounted on slides with 80% glycerol. For maximal Hh signal strength, a UAST-Hh construct was also included in the transfection (Fan et al., 2012).

Fluorescence signals were acquired with the 60× objective on an Olympus confocal microscope. Smo density was analyzed with ImageJ software (NIH, version 1.48v).

For the ptc-luc reporter assay, S2 cells were cultured in six-well plates and transfected with 50 ng tub-Ci, 20 ng renilla and 150 ng ptc-luc reporter vectors. At 48 h post-transfection, cells were lysed for luciferase activity analysis with the dual-luciferase reporter assay system (Promega, Madison, WI). Renilla luciferase was used to normalize the luciferase activity. The measurements of dual-luciferase were determined with a GLOMAX Multi Detection System (Promega).

Presented data are representative of three experiments, with standard deviation (s.d.) bars generated from four replicates. For statistical analysis, Student’s t-test was used for cell culture studies involving two independent groups.

Antibodies used in this study for western blotting were: mouse anti-Myc (1:5000; 9E10, Santa Cruz Biotechnology), anti-HA (1:1000; F7, Santa Cruz Biotechnology), anti-FLAG (1:500; 9E10, Santa Cruz Biotechnology), anti-GRP (1:1000; 25D11, Cell Signaling), rabbit anti-Myc (1:2000; A-14, Santa Cruz Biotechnology), anti-SMoP (1:50; Fan et al., 2012), and anti-Krz (1:2500; PA1-730, Thermo Scientific).

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