Cell membrane and subcellular localization of Smoothened in Hedgehog signaling

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Short Abstract:
Hedgehog (Hh) stimulation promotes Smo phosphorylation and accumulation on the cell surface. In this study, we describe the cell based assays to examine the cell membrane localized Smo and to monitor Smo subcellular trafficking that is regulated by Hh. Endosome markers are used to mark the subcellular localization of Smo.

Long Abstract:
The seven transmembrane protein Smoothened (Smo) is a critical component in the receptor complex of Hedgehog (Hh) signaling. Hh induces the cell-surface accumulation and phosphorylation of Smo by multiple kinases, including protein kinase A (PKA), casein kinase 1 (CK1), casein kinase 2 (CK2), and G protein-coupled receptor kinase 2 (GRK2). In this study, we demonstrate the methods to examine Smo cell-surface and subcellular localization. We show that the Hh stimulation promotes Smo cell-surface accumulation that is monitored by the Smo antibody staining. We further show that Hh prevents Smo internalization in an antibody uptake experiment in which Rab5 and Rab7 are used to label the early endosome and late endosome, respectively. We also show that, in the absence of Hh, Smo is presumably localized in the endosomes. Our data suggest that Smo intracellular trafficking is a highly regulated event that mediates the cellular response to ligand stimulation.

Protocol Text:
1.) Cell-Surface staining
1.1) cultured *Drosophila* S2 cells at 25˚C on sterile cover slips in 6-well plate were transfected with UAST-CFP-Smo (0.2μg/well), using Effectene transfection reagent. An Ub-Gal4 cDNA construct (0.15μg/well) was cotransfected to drive the expression of UAST-CFP-Smo.

1.2) 24 hours after transfection, cell culture medium were changed into either control medium or medium containing 60% Hh-conditioned medium for 24 hours (Jia et al., 2004).

1.3) transfected S2 cells were washed 3 times (10 minutes/wash) with 1xPBS (1ml /well), followed by fixation with 4% formaldehyde in PBS (1ml/well) for 20 minutes.

1.4) cells were then washed with 1xPBS for 3 times (10 minutes/wash).

1.5) cell samples were blocked with 1.5% normal blocking serum in PBS for 30 minutes to prevent non-specific staining. Blocking serum ideally should be derived from the same species in which the secondary antibody is raised.

1.6) cells were incubated with the mouse anti-SmoN antibody (1:100) in 1% normal blocking serum for 2 hours before cell permeabilization (Jia et al., 2004). The SmoN antibody recognizes the extracellular domain of Smo (Lum et al., 2003).

1.7) cell samples were washed for 3 times with 1 ml 1xPBT (0.1% Triton in PBS, 10 minutes/wash) to allow cell permeabilization.

1.8) cell samples were incubated with Rhodamine-conjugated goat anti-mouse IgG (1:500) in 1 ml 1xPBT for 1 hour in dark chamber.

1.9) cell samples in 6-well plate were washed with 1 ml 1xPBT for 3 times (10 minutes/wash) in dark chamber.

1.10) cover slips were removed from the 6-well plate, mounted with the mounting medium, and fastened with fingernail polish on slides.

1.11) immunofluorecence was analyzed under the Olympus Fluoview 1000 confocal microscope. Rhodamine signals represent the cell surface localized Smo and CFP signals represent the total Smo that are expressed. Metamorph software was used to calculate the ratio of the cell surface-localized Smo vs. total Smo. See Figure 1.

2.) Antibody uptake

2.1) cultured S2 cells on sterile coverslips in 6-well plate were transfected with UAST-CFP-Smo (0.2μg /well) either alone or along with UAST-RFP-Rab7 (0.15μg/well), using Effectene transfection reagent. RFP-Rab7 was constructed by sub-cloning one copy of the Red Fluorescent Protein (RFP) in frame to the N-terminus of Rab7 in the backbone of UAST vector. Rab7 was used to label the later endosome. Ub-Gal4 cDNA construct (0.15μg/well) was cotransfected to drive the expression of the target proteins.

2.2) 24 hours after transfection, cells were treated with 60% Hh-conditioned medium or control medium and then incubated in these medium for the continued 24 hours.

2.3) the mouse anti-Smo antibody (concentrated, 1:100) was added to the living culture in normal growth condition for 2 hours in order to allow the cells to uptake the antibody.

2.4) cells in 6-well plate were washed with 1 ml 1xPBS for 3 times (10 minutes/wash), then fixed with 4% formaldehyde in 1xPBS (1 ml/well) for 20 minutes.

2.5) the coverslips in 6-well plate were washed with 1 ml 1xPBT for 3 times (10 minutes/wash) to allow cell permeabilization.

2.6) to label the endogenous Rab5, cells on cover slips were stained with rabbit anti-Rab5 antibody (1:500) at room temperature for 2 hours. Rab7 could be monitored by auto-flourecence when UAST-RFP-Rab7 was cotransfected.

2.7) samples on coverslips in 6-well plate were then washed with 1 ml 1xPBT for 3 times (10 minutes/wash).

2.8) cell samples were incubated with Cy5-conjugated goat anti-rabbit IgG and Rhodamine-conjugated goat anti-mouse IgG in 1xPBT for 1 hour in dark chamber.

2.9) samples were washed with 1 ml 1xPBT for 3 times (10 minutes/wash) in dark chamber.

2.10) coverslips were removed from the 6-well plate, mounted with the mounting medium, and fastened with fingernail polish on slides.

2.11) immunofluorecence was analyzed under the Olympus Fluoview 1000 confocal microscope. Rhodamine signals represent the uptaken Smo. CFP signals represent the total Smo that are expressed. Cy5 or RFP signals label the early or late endosomes in the cytosal. Z-scan sections of regular cells (12-14 μm in diameter) were taken at 0.7 μm by confocal imaging to monitor the Rab5-positive early endosomes and the Rab7-positive late endosomes and to quantify the amount Smo that was endocytosed after intensity projection over the Z axis. For quantification, an autoradiography densitometric analysis was performed using Metamorph software. Where appropriate, the experimental groups were compared using the Student's two-tailed *t* test, with significance defined as $P<0.05$.

Tables and Figures:

Figure 1 Smo cell-surface staining. (A-B'') CFP-Smo was transfected into S2 cells followed by the treatment with Hh-conditioned medium or control medium. Smo that localized to the cell surface was visualized by immunostaining with an anti-SmoN antibody before membrane permeabilization. The total amount of expressed Smo was indicated

by the CFP signal. (C) Quantification analysis of the percentage of Smo on the cell surface (mean ± s.d.; n ≥ 15). Ratio (%) = (cell surface signal/whole cell signal) x 100.

Figure 2 SmoN antibody uptake staining. (A) A drawing illustrating the antibody uptake procedures. The red arrowheads indicate the route of the endocytosed anti-SmoN antibody. (B-C) S2 cells were transfected with CFP-Smo and treated with control medium or Hh-conditioned medium. The images shown here are from intensity projection over the Z axis. (D-E) S2 cells were cotransfected with CFP-Smo and RFP-Rab7 and treated with control medium or Hh-conditioned medium. Again, shown here are image projections over the Z axis. (F) The percentage of Rab5-colocalized Smo in the absence or presence of Hh. Results were calculated from B-C (n≥ 10). (G) The percentage of Rab5-colocalized Smo in the absence or presence of Hh. Results were calculated from D-E (n≥ 10).

Discussion:

Smo is a G protein-coupled receptor (GPCR) like membrane protein that acts as a key regulator in the Hh reception system. Hh induces the cell surface accumulation and phosphorylation of Smo (Denef et al., 2000) by multiple kinases, including protein kinase A (PKA),casein kinase 1 (CK1), casein kinase 2 (CK2), and G protein-coupled receptor kinase 2 (GRK2) (Apionishev et al., 2005; Chen et al., 2010; Jia et al., 2010; Jia et al., 2004; Zhang et al., 2004), which activate Smo by inducing a conformational change in the protein (Zhao et al., 2007). It has been shown that forced localization of Smo to the cell surface increases signaling activity, whereas endoplasmic retention of an activated form of Smo blocks activity (Zhu et al., 2003). The inhibition of endocytosis through the use of a dominant-negative form of Rab5 promotes Smo cell surface accumulation (Zhu et al., 2003). Notably, Smo is primarily localized to the lysosomes of A-compartment cells in *Drosophila* imaginal discs, where Hh is not present, and is enriched on the plasma membrane of P-compartment cells where Hh stimulation occurs (Nakano et al., 2004). Similar mechanisms have been proposed for the regulation of mammalian Smo, whereby both Smo and Ptc co-localize and internalize in endosomal compartments, and Hh induces the segregation of Smo away from Hh-Ptc complexes that are destined for lysosome degradation (Incardona et al., 2002). In addition, the association of Smo with β-arrestin 2 appears to promote Smo endocytosis through clathrin-coated pits (Chen et al., 2004). Taken together, the controlled accumulation and localization of Smo in the Hh signaling pathway is thought to play a central role in maintaining signaling homeostasis. However, it is yet unclear how Hh controls the intracellular trafficking of Smo (Jiang and Hui, 2008).

This study describes the methods with which Smo cell-surface accumulation and intracellular trafficking are examined. We sought to understand the cellular mechanisms by which Hh promotes Smo accumulation on the cell surface.

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Disclosures: We have nothing to disclose

Table of specific reagents and equipment:

Name of the reagent	Company	Catalogue number	Comments (optional)
Formadehyde	Sigma	36996JM	
6-well plate	Greinerbio-one	E1011011	
Schneider's Drosophila medium	GIBCO	11720	
FBS	Sigma	12303-C	
Effectene transfection reagent	Qiagen	301427	
Cover slips	Fisher Scientific	12-548-A	
slides	Fisher Scientific	12-550-123	

Anti-Rab5	AbCam	Ab31261	
Anti-SmoN	DHSB	20C6	
Rhodamine-conjugated goat anti-mouse IgG	Jackson Laboratory		
Cy5-conjugated goat anti-rabbit IgG	Jackson Laboratory		
Mounting medium	Vector laboratory	H-100	
Fingernail polish	Scherer Inc	N/A	
Confocal microscope	Olympus	Fluview 1000 Ver.1.7c	

References:

Apionishev, S., Katanayeva, N.M., Marks, S.A., Kalderon, D., and Tomlinson, A. (2005). Drosophila Smoothened phosphorylation sites essential for Hedgehog signal transduction. Nat Cell Biol *7*, 86-92.

Chen, W., Ren, X.R., Nelson, C.D., Barak, L.S., Chen, J.K., Beachy, P.A., de Sauvage, F., and Lefkowitz, R.J. (2004). Activity-dependent internalization of smoothened mediated by beta-arrestin 2 and GRK2. Science *306*, 2257-2260.

Chen, Y., Li, S., Tong, C., Zhao, Y., Wang, B., Liu, Y., Jia, J., and Jiang, J. (2010). G protein-coupled receptor kinase 2 promotes high-level Hedgehog signaling by regulating the active state of Smo through kinase-dependent and kinase-independent mechanisms in Drosophila. Genes Dev *24*, 2054-2067.

Denef, N., Neubuser, D., Perez, L., and Cohen, S.M. (2000). Hedgehog induces opposite changes in turnover and subcellular localization of patched and smoothened. Cell *102*, 521-531.

Incardona, J.P., Gruenberg, J., and Roelink, H. (2002). Sonic hedgehog induces the segregation of patched and smoothened in endosomes. Curr Biol *12*, 983-995.

Jia, H., Liu, Y., Xia, R., Tong, C., Yue, T., Jiang, J., and Jia, J. (2010). Casein kinase 2 promotes Hedgehog signaling by regulating both smoothened and Cubitus interruptus. Journal Of Biological Chemistry *285*, 37218-37226.

Jia, J., Tong, C., Wang, B., Luo, L., and Jiang, J. (2004). Hedgehog signalling activity of Smoothened requires phosphorylation by protein kinase A and casein kinase I. Nature *432*, 1045-1050.

Jiang, J., and Hui, C.C. (2008). Hedgehog signaling in development and cancer. Dev Cell *15*, 801-812.

Lum, L., Zhang, C., Oh, S., Mann, R.K., von Kessler, D.P., Taipale, J., Weis-Garcia, F., Gong, R., Wang, B., and Beachy, P.A. (2003). Hedgehog signal transduction via Smoothened association with a cytoplasmic complex scaffolded by the atypical kinesin, Costal-2. Mol Cell *12*, 1261-1274.

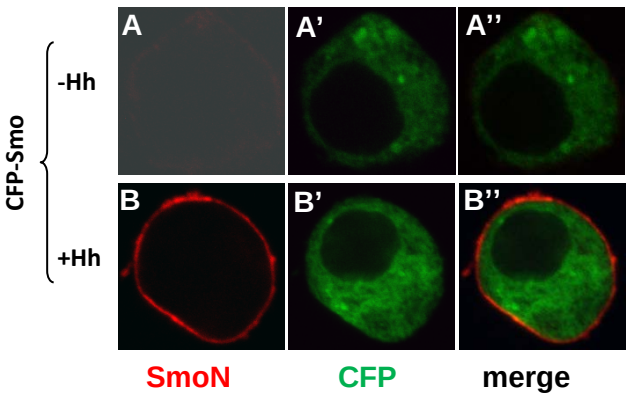
Nakano, Y., Nystedt, S., Shivdasani, A.A., Strutt, H., Thomas, C., and Ingham, P.W. (2004). Functional domains and sub-cellular distribution of the Hedgehog transducing protein Smoothened in Drosophila. Mechanisms Of Development *121*, 507-518.

Zhang, C., Williams, E.H., Guo, Y., Lum, L., and Beachy, P.A. (2004). Extensive phosphorylation of Smoothened in Hedgehog pathway activation. Proceedings Of The National Academy Of Sciences Of The United States *101*, 17900-17907.

Zhao, Y., Tong, C., and Jiang, J. (2007). Hedgehog regulates smoothened activity by inducing a conformational switch. Nature *450*, 252-258.

Zhu, A.J., Zheng, L., Suyama, K., and Scott, M.P. (2003). Altered localization of Drosophila Smoothened protein activates Hedgehog signal transduction. Genes Dev *17*, 1240-1252.

Fan_Fig. 1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+Hh
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A'
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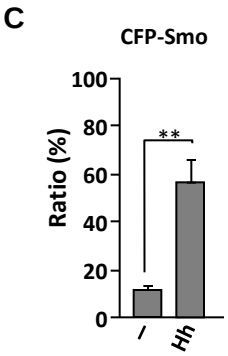
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CFP-Smo
Ratio (%)
100
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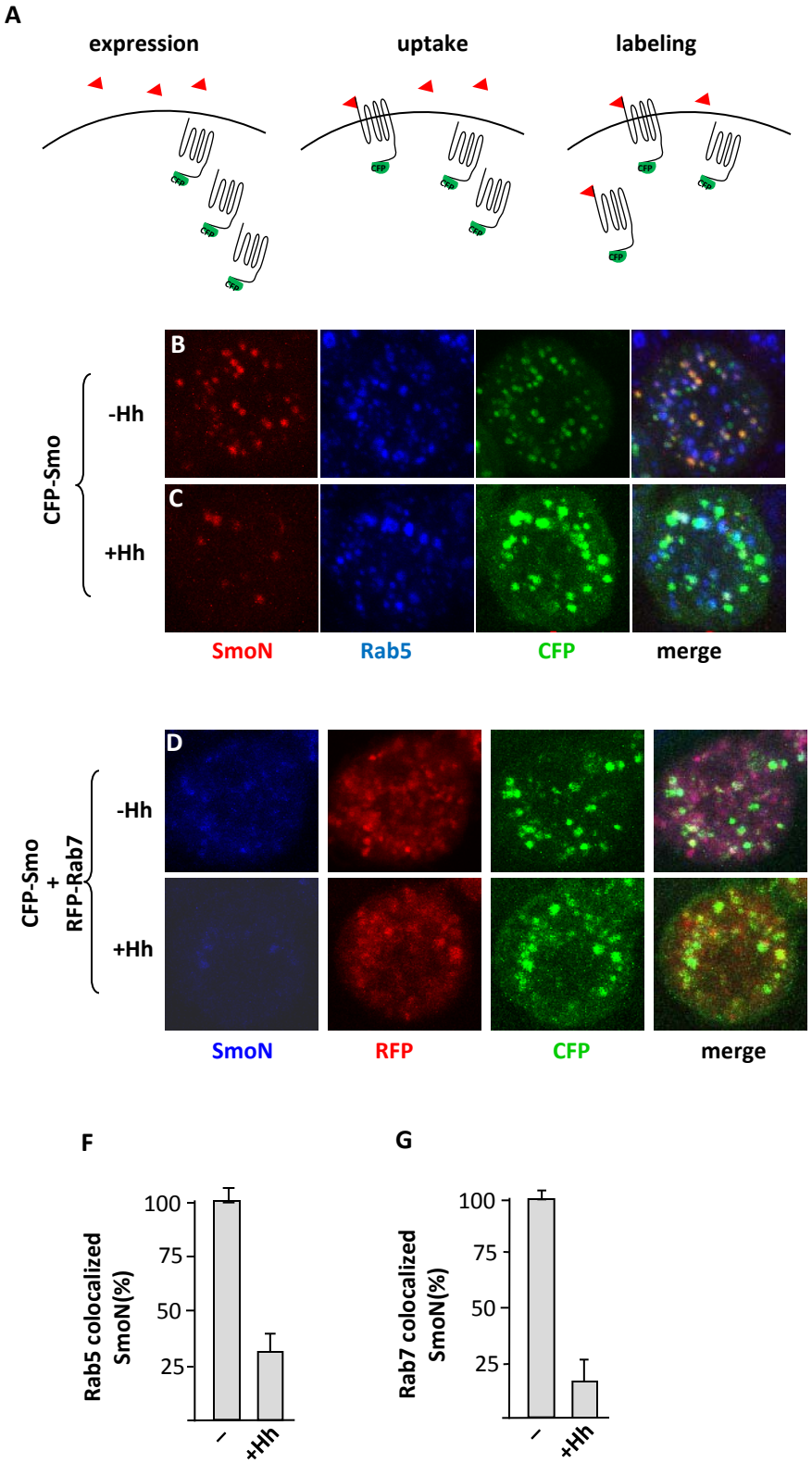
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SmoN
RFP
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Rab5 colocalized SmoN(%)
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Rab7 colocalized SmoN(%)
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