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Ariel H. Polizio, Pilar Chinchilla, Xiaolen Chen, David R. Manning and Natalia A. Riobo (22 November 2011)

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Sonic Hedgehog Activates the GTPases Rac1 and RhoA in a Gli-Independent Manner Through Coupling of Smoothened to G_i Proteins

Ariel H. Polizio,[1] Pilar Chinchilla,[1] Xiaolen Chen,[1] David R. Manning,[2] Natalia A. Riobo[1,3]*

The vertebrate Hedgehog (Hh) pathway has essential functions during development and tissue homeostasis in normal physiology, and its dysregulation is a common theme in cancer. The Hh ligands (Sonic Hh, Indian Hh, and Desert Hh) bind to the receptors Patched1 and Patched2, resulting in inhibition of their repressive effect on Smoothened (Smo). Smo is a seven-transmembrane protein, which was only recently shown to function as a G protein–coupled receptor (GPCR) with specificity toward the heterotrimeric guanine nucleotide-binding protein G_i. In addition to activating G_i, Smo signals through its C-terminal tail to inhibit Suppressor of Fused, resulting in stabilization and activation of the Gli family of transcription factors, which execute a transcriptional response to so-called "canonical Hh signaling." In this Presentation, we illustrate two outcomes of Hh signaling that are independent of Gli transcriptional activity and, thus, are defined as "noncanonical." One outcome is dependent on Smo coupling to G_i proteins and exerts changes to the actin cytoskeleton through stimulation of the small guanosine triphosphatases (GTPases) RhoA and Rac1. These cytoskeletal changes promote migration in fibroblasts and tubulogenesis in endothelial cells. Signaling through the other noncanonical Hh pathway is independent of Smo and inhibits Patched1-induced cell death.

Presentation Notes

Slide 1: Science Signaling *logo*

The slideshow and notes for this Presentation are provided by *Science Signaling* (http://www.sciencesignaling.org).

Slide 2: Sonic Hedgehog activates the GTPases Rac1 and RhoA in a Gli-independent manner through coupling of Smoothened to G_i proteins

This Presentation demonstrates that mammalian Hedgehog (Hh) signaling is not limited to those events leading to Gli-mediated transcriptional changes. We present evidence that Hh ligands induce important changes in the actin cytoskeleton that modulate cell motility and morphology through stimulation of the small guanosine triphosphatases (GTPases) Rac1 and RhoA in a manner that is dependent upon the heterotrimeric G protein G_i.

[1]Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA 19107, USA. [2]Department of Pharmacology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA. [3]Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA.

*Presenter and corresponding author. E-mail, natalia.riobo@jefferson.edu

Slide 3: Determination of Smo-G protein coupling

The most direct assay for determining activation of a heterotrimeric G protein (Gαβγ) by a purported G protein–coupled receptor (GPCR), which is commonly referred to as "coupling," is the quantification of guanosine triphosphate (GTP) binding to the Gα subunit. For this assay, we expressed the seven-transmembrane protein Smoothened (Smo) together with different Gα subunits representing the four families (G_s, G_i, G_q, and $G_{12/13}$) and Gβ1γ2 in cultured insect Sf9 cells by means of baculoviral transduction. This allows for isolation of membranes highly enriched in Smo with specific combinations of G proteins. If Smo couples to a particular Gαβγ combination, it will increase the rate of guanosine diphosphate (GDP) release, which is quickly replaced by excess [^{35}S]guanosine 5′-(3-*O*-thio)triphosphate ([S^{35}]GTPγS) present in the reaction mix. Proteins are rapidly solubilized under nondenaturing conditions, and Gα is immunoprecipitated with specific antisera. After extensive washing, [S^{35}]GTPγS bound to Gα is quantified by liquid scintillation, as described (*1*).

Slide 4: Smo is a G_i-selective GPCR

Using the GTP binding assay described on the previous slide, we observed that Smo stimulates GTP incorporation in the α subunits of all members of the G_i family tested ($G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_o$, and $G\alpha_z$), as shown by the red bars (*2*). The black bars represent spontaneous GTP incorporation when the G proteins are expressed in the absence of Smo. Importantly, Smo was unable to couple to G proteins of the G_s, G_q, and G_{12} families (aquamarine bars). In mammals, most G_i members are sensitive to inhibition by pertussis toxin (PTX), which adenosine diphosphate (ADP)–ribosylates a Cys residue in the Gα subunit. The only exception to this sensitivity is $G\alpha_z$, which is almost exclusively expressed in nervous tissue and platelets (*3*).

Slide 5: Modulation of Smo-G_i coupling by small agonists and antagonists of Smo

The Smo-G_{i2} pair was used to determine whether the constitutive activity displayed by Smo can be modulated by small molecules that alter Gli transcriptional activity by binding to Smo. As shown in the left panel, addition of the Smo agonist purmorphamine (*4*) increased G_{i2} activation in a concentration-dependent manner, with a median effective concentration (EC_{50}) of 1.4 μM. The Smo inhibitors cyclopamine, 3-keto-n-aminoethyl-aminocaproyl-dihydrocinnamoyl (KAAD)–cyclopamine, and SANT-1 were able to dose-dependently reduce GTP incorporation to an amount comparable to that of G_{i2} alone and with median inhibitory concentrations (IC_{50}'s) similar to those reported for Gli transcription inhibition in cell-based assays (80 nM, 200 nM, and 15 nM, respectively) (*5*, *6*). The inactive cyclopamine analog tomatidine did not affect GTP incorporation at any concentration. These observations also suggest that the three Smo inhibitors behave pharmacologically as inverse agonists.

Slide 6: Noncanonical Hedgehog signaling

We define as "noncanonical" Hh signaling those events that occur in response to bind-

ing of a Hh ligand to its receptors Patched1 (PTCH1) or Patched2 (PTCH2) and are independent of Gli-dependent transcriptional regulation. Mechanistically, there are at least two types of noncanonical Hh signaling. The first class (Type I) depends on Patched but is not exerted through modulation of Smo's activity and, therefore, is insensitive to cyclopamine and not mimicked by Smo agonists. The second class (Type II) is dependent on Smo and mediates cyclopamine-sensitive cellular responses independent of Gli activation. Because Smo couples to G_i, we hypothesized that at least some Type II mechanisms will depend on Smo-G_i coupling and, therefore, be sensitive to inhibition by PTX.

Slide 7: Type II noncanonical Hh signaling paradigm

We decided to study the role of Smo as a G_i-coupled receptor in fibroblast migration stimulated by Sonic Hedgehog (Shh) in a Gli-independent manner (*7*).

Slide 8: Fibroblast migration: Genetic models and canonical pathway status

We used NIH 3T3 fibroblasts and mouse embryonic fibroblasts (MEFs) of different genotypes: cells heterozygous for a null allele of *Patched1* (*Ptc1*$^{+/-}$), which are functionally identical to wild-type (WT) cells and are responsive to Shh stimulation; cells homozygous for a null allele of *Patched1* (*Ptc1*$^{-/-}$), in which the canonical Hh pathway is constitutively activated; and cells homozygous for a null mutation of *Smo*, (*Smo*$^{-/-}$), in which Glis cannot be activated in response to Shh stimulation.

Slide 9: Shh promotes fibroblast migration in a Smo-dependent manner

Addition of Shh to serum-starved WT fibroblasts or *Ptc1*$^{+/-}$ MEFs increased the rate of migration in response to a scratch in the monolayer (micrographs and black bars) (*8*). The direct Smo agonist purmorphamine (PUR) stimulated cell migration to a similar extent as Shh. The increase in motility induced by Shh or PUR was abolished by addition of the Smo inhibitor KAAD-cyclopamine (white bars) (*8*).

Slide 10: Smo activity promotes fibroblast migration

In the absence of Shh, the spontaneous rate of migration of *Ptc1*$^{-/-}$ MEFs is significantly higher than that of *Ptc1*$^{+/-}$ cells ($P = 0.023$) (panel A). This difference can be attributed entirely to the unrepressed activity of Smo, because addition of KAAD-cyclopamine (denoted as KAAD or KAAD-CP) reduces the spontaneous rate to that of *Ptc1*-expressing cells (panel A). In support for an essential role of Smo in migration, the *Smo*-deficient MEFs are insensitive to a Smo agonist (PUR) and a Smo inverse agonist (KAAD) (panel B). These results confirm that activation of Smo is necessary and sufficient to promote fibroblast migration (*8*). Because this signaling paradigm is active in *gli2*$^{-/-}$*; gli3*$^{-/-}$ MEFs, which do not show evidence of any Gli-dependent transcriptional activity (*7*), it is likely to be a Type II noncanonical response.

Slide 11: Smo signaling drives fibroblast migration in a G_i- and PI3K-dependent manner

Noncanonical signaling downstream of Smo may involve activation of G_i or modulation of Sufu activity by the cytoplasmic C-terminal tail of Smo, or both. To address whether Smo promotes cell migration in a G_i-dependent manner, we pretreated the cells with PTX before PUR treatment. Inhibiting G_i activation completely abolished the effect of PUR (panel A), indicating that coupling of Smo to G_i is essential for signaling through the Type II noncanonical Hh pathway.

We had previously reported that phosphoinositide 3-kinase (PI3K) activity is essential for canonical Hh signaling (*9*). Because the γ isoform of PI3K (PI3Kγ) can be activated by free Gβγ subunits and is involved in cell motility, we reasoned that activation of PI3K downstream of G_i might play a role in Shh-induced migration. As predicted, the PI3K inhibitor LY294002 (denoted LY) prevented the increased motility of PUR-treated cells (panel A). These data indicate that fibroblast migration induced by acute activation of Smo is dependent on G_i proteins and PI3K.

In support of the proposed mechanism, the increased basal migration observed in *Ptc1*$^{-/-}$ cells by constitutive activation of Smo is also mediated by G_i and PI3K, and the low motility of *Smo*-deficient MEFs is insensitive to PTX and LY294002 (panel B). *Ptc1*$^{-/-}$ MEFs also have higher amounts of the phosphorylated forms of the PI3K targets 3-phosphoinositide-dependent kinase 1 (PDK1) and v-Akt murine thymoma viral oncogene homolog 1 (Akt1) under serum starvation conditions as compared with *Smo*$^{-/-}$ MEFs. This increase is due to increased Smo-G_i signaling, because it is abolished by treatment with KAAD-cyclopamine or PTX (right panels).

Slide 12: Shh activates the small GTPases RhoA and Rac1

Cell motility occurs by the coordinated action of small GTPases of the Rho family, which modulate actin cytoskeleton dynamics. Addition of Shh to fibroblasts induced a fast activation of both Rac1 (panel A) and RhoA (panel B), as determined using pull-down assays, with different kinetics. Introduction of dominant negative mutant versions of the small G proteins RacN17 and RhoN19 blocked Shh-induced migration and reduced basal motility as well (panels C and D).

Slide 13: Smo controls RhoA and Rac1 activation

Remarkably, serum-starved *Ptc1*$^{-/-}$ cells show a very high basal activity of RhoA (panel A) and Rac1 (panel B), comparable to that of *Ptc1*$^{+/-}$ cells after stimulation with Shh. Addition of KAAD-cyclopamine reduced the basal activation of these small GTPases in *Ptc1*$^{-/-}$ MEFs, demonstrating that their activation is driven by unrepressed Smo.

Slide 14: Activation of RhoA and Rac1 by Shh is mediated by G_i and PI3K

Because Smo coupling to G_i mediates cell migration in response to Shh and Smo activates RhoA and Rac1, we sought to evaluate whether G_i proteins were necessary intermediaries for activation of the small GTPases. Pretreatment of NIH3T3 cells with PTX abolished Shh-induced stimulation of RhoA (panel A) and Rac1 (panel B). Moreover, the PI3K inhibitor LY294002 also prevented activation of RhoA and Rac1, as was shown before for Shh-induced migration. These observations suggest that G_i and PI3K link Smo to the actin cytoskeleton.

Slide 15: Activation of Rac1 by Shh does not require Gli activity

Although the timing of Rac1 activation suggested that it was not a transcriptional effect, we decided to completely rule out a possible contribution of the canonical Hh pathway to activation of the small GTPases and to fibroblast migration. To this end, we transduced *Ptc1*$^{+/-}$ MEFs transfected with an adenovirus encoding green fluorescent protein (GFP) alone or a GFP-tagged version of the 83-kD Gli3 repressor (Gli3R-GFP). Expression of Gli3R-GFP did not impair Rac1 activation (panel A) or fibroblast migration (panel B), confirming that these cellular responses to Shh are noncanonical.

Slide 16: The C-terminal tail of Smo is required for activation of Gli, but not for coupling to G_i

We had previously demonstrated that Smo lacking the C-terminal tail (SmoΔC), which is recognized by an N-terminal–directed antibody (N-19) but not by a C-terminal–

targeted antibody (H-300) (panel A), retains full coupling activity toward G_{i1} and G_{i2} (panel B) and that this coupling is sensitive to cyclopamine (panel C). However, deletion of the C-terminal tail abrogated Smo's ability to stimulate a Gli-responsive luciferase reporter in NIH3T3 cells, even if it carries the oncogenic mutation W539L that confers constitutive activity (SmoM2ΔC) (panel D).

Slide 17: Which activity of Smo is required for cell migration and Rho GTPase regulation?

Independent proof that Smo coupling to G_i is a separate function from activation of the Gli transcription factors came from rescue experiments in *Smo*-deficient MEFs. We reintroduced the full-length constitutively active SmoM2, a mutant in which the WR motif that is required for ciliary localization was mutated to AA (SmoM2-CLD), or a C-terminal–deleted version of this mutant (SmoM2ΔC) into $Smo^{-/-}$ cells. Only SmoM2 and SmoM2-CLD were able to stimulate the canonical Hh pathway 24 hours after reaching high cell density in culture (panel A). Rescue by SmoM2-CLD was unexpected because translocation to the primary cilium is a requirement for activation of the canonical pathway (*10*). However, the M2 mutation might conceivably overcome the need for the WR motif for ciliary accumulation. Regardless, SmoM2ΔC did not restore canonical Hh signaling but selectively rescued activation of Rac1 (panel B), RhoA (panel C), and cell migration (panel D) in $Smo^{-/-}$ MEFs, demonstrating that coupling of Smo to G_i is not only necessary but sufficient to engage this noncanonical mode of Hh signaling.

Slide 18: All Hh isoforms promote tubulogenesis in endothelial cells

Another example of Type II noncanonical signaling has been reported by our group in an endothelial cell tubulogenesis model (*11*). Endothelial cells cultured in three-dimensional (3D) matrices have an intrinsic tendency to rearrange into tube-like structures through a process known as tubulogenesis. Addition of Shh, Indian Hh (Ihh), or Desert Hh (Dhh) to a 3D culture of endothelial cells in matrigel or collagen Type I increased both the number and branching of tubes (panels A and B). This effect was strictly dependent on Smo and G_i, because it was inhibited by KAAD-cyclopamine and PTX (panel B). Stimulation of endothelial cells with any of the three Hh ligands increased the abundance of active RhoA (panel C) in a Smo- and G_i-dependent manner (panel D), akin to what we observed in fibroblasts.

Slide 19: Noncanonical Type II Hh pathway: Linking Hh to small GTPases

We have provided here two examples of the existence of a Gli-independent function of the Hh family of proteins that involves sequential activation of Smo, G_i, and small GTPases of the Rho family to promote remodeling of the actin cytoskeleton. In fibroblasts this pathway is linked to migration, whereas in endothelial cells it promotes tubulogenesis (a proangiogenic function). Mechanistically, in these contexts Smo activates G_i and PI3K to trigger RhoA and Rac1 activation independent of its cytoplasmic C-terminal tail, which is essential for canonical signaling.

Slide 20: Type I noncanonical Hh pathway: Inhibiting PTCH1–initiated apoptosis

In the next series of slides, we present evidence for a different type of noncanonical Hh signaling that depends exclusively on Ptch1 and regulates apoptosis in endothelial cells.

Slide 21: Shh, Ihh, and Dhh reduce apoptosis induced by serum starvation in endothelial cells

Endothelial cells are believed to be unresponsive to Hh because there is no reported evidence of Gli transcriptional activity in these cells either in vitro or in vivo (*11*–*13*). We have confirmed that human umbilical vein endothelial cells (HUVECs) and human microvascular endothelial cells (HMVECs) are incapable of activating the canonical Hh pathway (*11*). In addition, none of the three Hh isoforms stimulated proliferation in these cells (panel A). Nonetheless, Shh, Ihh, and Dhh were equally effective in reducing caspase-3 activation, which is a measure of apoptosis, after serum withdrawal (panel B).

Slide 22: Shh antiapoptotic activity is independent of Smo

The reduction in caspase-3 activity by administration of Shh was insensitive to any of three Smo inhibitors: SANT-1, cyclopamine (CP), and KAAD-cyclopamine (KAAD). Moreover, the direct Smo agonist purmorphamine did not decrease caspase activation as Hh proteins do. Thus, modulation of Smo activity is not involved in the Shh antiapoptotic activity, suggesting that this activity is mediated by a Type I noncanonical pathway.

Slide 23: Shh inhibits endothelial cell apoptosis by inhibition of PTCH1

It has been reported that overexpression of PTCH1 in human cells induces apoptosis by recruiting a protein complex that contains caspase-9 (*14*, *15*). To determine whether Shh reduces apoptosis in endothelial cells by inhibiting PTCH1, we depleted PTCH1 in HUVEC cells by 80 to 85% using two different small interfering RNA (siRNA) duplexes (P1 and P2) (panel A). An siRNA dose-dependence experiment revealed a direct correlation between the abundance of PTCH1 and caspase-3 activity in the absence of serum (panel B). Depletion of PTCH1 was also accompanied by an increase in cell number, which was not the result of unrepressed Smo, because KAAD-cyclopamine could not block the effect of PTCH1 depletion (panel C). In addition, Shh was unable to modulate caspase-3 activity in cells in which PTCH1 had been depleted (panel D), indicating that the antiapoptotic, prosurvival role of Shh in endothelial cells is exerted by inhibition of the proapoptotic function of PTCH1 through a Type I signaling pathway.

Slide 24: Acknowledgments

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Editor's Note: This contribution is not intended to be equivalent to an original research paper. Note, in particular, that the text and associated slides have not been peer-reviewed.

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