Role of Lipid Metabolism in Smoothened Derepression in Hedgehog Signaling

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SUMMARY

The binding of Hedgehog (Hh) to its receptor Patched causes derepression of Smoothened (Smo), resulting in the activation of the Hh pathway. Here, we show that Smo activation is dependent on the levels of the phospholipid phosphatidylinositol-4-phosphate (PI4P). Loss of STT4 kinase, which is required for the generation of PI4P, exhibits hh loss-of-function phenotypes, whereas loss of Sac1 phosphatase, which is required for the degradation of PI4P, results in hh gain-of-function phenotypes in multiple settings during Drosophila development. Furthermore, loss of Ptc function, which results in the activation of Hh pathway, also causes an increase in PI4P levels. Sac1 functions downstream of STT4 and Ptc in the regulation of Smo membrane localization and Hh pathway activation. Taken together, our results suggest a model in which Ptc directly or indirectly functions to suppress the accumulation of PI4P. Binding of Hh to Ptc derepresses the levels of PI4P, which, in turn, promotes Smo activation.

INTRODUCTION

The Hedgehog (Hh) signaling pathway, which was discovered through genetic analysis during Drosophila development, is highly conserved across evolution and functions in a diverse array of developmental decisions (Jiang and Hui, 2008). The embryonic functions of the Hh pathway are recapitulated in the adult, where it is required for the maintenance of stem cell fate and for tissue repair (Beachy et al., 2004). Consistent with its diverse role, loss of Hh signaling results in developmental defects, and its overactivation has been implicated in the etiology of cancer (Jiang and Hui, 2008).

Activation of the signaling cascade occurs upon binding of Hh to its receptor, the 12 trans-membrane protein Patched (Ptc), which differs from conventional receptors for intercellular signals in that it functions as a pathway inhibitor (Hooper and Scott, 1989; Jiang and Hui, 2008; Stone et al., 1996). Upon ligand binding, Ptc is inactivated, which in turn leads to a relief of repression of Smoothened (Smo), a seven trans-membrane protein similar to G-coupled receptors, that transduces the signal inside the cell (Alcedo et al., 1996; van den Heuvel and Ingham, 1996). The specific details of the intracellular relay mechanism activated by Smo differs somewhat between vertebrates and flies but culminates in the activation of a transcriptional effector, Cubitus Interruptus (Ci), in Drosophila and a group of GlI proteins in vertebrates (Ruíz i Altaba et al., 2002). In the absence of the Hh signal, the full-length (155 kDa) Ci protein is processed by the proteasome-ubiquitin pathway to generate a 75 kDa product that functions as a repressor of Hh pathway target genes. Upon activation of the signal, the proteasome-ubiquitin-mediated processing of Ci is blocked and full-length Ci is stabilized and migrates to the nucleus, functioning as an activator of Hh target genes. Ptc, the receptor for Hh, and also the negative regulator of the pathway, is transcriptionally up-regulated by Ci, setting up a feedback loop to control the amplitude and duration of the signal.

A major unresolved issue in the Hedgehog signal transduction pathway is the mechanism by which Ptc inhibits Smo to block the activation of the pathway, and this issue constitutes a primary focus of this study. Biochemical analysis suggests that Smo can exist either in an active or an inactive state and that Ptc down-regulates Smo function by stabilizing the inactive state of Smo. This process appears to be catalytic, rather than through stoichiometric interactions, because Ptc inhibits Smo even when the latter is present in molar excess (Taipale et al., 2002). As a further complication, in spite of multiple binding studies, Ptc has never been found to be physically associated with Smo (Denef et al., 2000; Taipale et al., 2002). Genetic studies in flies suggest that Ptc regulates the membrane localization of Smo, and, in ptc mutants, Smo is localized constitutively to the membrane, activating the pathway. Likewise, in vertebrate model systems, localization of Smo to the primary cilium of the cell is essential for Hh pathway activation, and Ptc inhibits its transport to these primary cilia (Huangfu et al., 2003; Rohatgi et al., 2007). Finally, the Ptc–Smo interaction appears to be one of the most important steps in the Hh pathway because it is one of the most frequently disrupted steps in Hh-pathway-related human cancers (Rohatgi and Scott, 2007).
Apart from Ptc-mediated relief of repression, Smo activation has also been proposed to require the binding of a small molecule, rather than a protein ligand. Cyclopamine, a plant alkaloid that is teratogenic in sheep, binds Smo and inhibits its function. Likewise, studies in tissue culture cells suggest that sterollike molecules can bind Smo, and also the Ptc protein has a sterol sensor domain, a 180 amino acid module present in proteins involved in sterol metabolism and vesicular transport (Eaton, 2008). Finally, sequence homology studies suggest that Ptc protein shows similarity to the Bacterial Resistance, Nodulation Division family of proteins, which function as homotrimERIC small molecule pumps (Taipale et al., 2002). In this scenario, Ptc is proposed to function as a pump to change the concentration of a small molecule involved in Smo activation. The nature of the proposed small molecule and its relationship to the Ptc/ SMO interaction remains unresolved.

Phosphoinositols (PIs) are lipid constituents of the plasma and organelle membranes of all cells and occur as a collection of seven different phosphorylated versions. The interconversion between different PI moieties is regulated by multiple kinases and phosphatases (Blero et al., 2007; Skwarek and Boulianne, 2009). Different versions of PI show specific localization to membranes of different subcellular compartments and regulate cytoskeletal organization, signal transduction, and membrane and protein trafficking (Skwarek and Boulianne, 2009). In yeast, the generation of phosphatidylinositol-4 phosphate (PI4P), the first step in the synthesis of all PIs, is regulated by two genes, PIK1 and STT4, which are required for cell viability and are highly conserved across evolution (Audhya et al., 2000; Flanagan et al., 1993; Yoshida et al., 1994). These two genes function in a nonredundant manner because overexpression of STT4 in PIK1 mutant background does not rescue its phenotype and suggests that STT4 and PIK1 generate distinct nonoverlapping pools of PI4P (Foti et al., 2001). Localization studies further suggest that PIK1 is primarily present in the nucleus and the Golgi (Audhya et al., 2000; Walch-Solmena and Novick, 1999), whereas STT4 is primarily localized to the cytoplasm. In yeast, the cytoplasmic STT4 is recruited to the plasma membrane by trans-membrane proteins for localized synthesis of PI4P on the membrane and is required for PKC-1-dependent activation of the MAP kinase cascade (Audhya and Emr, 2002). Inhibition of the mammalian STT4 homolog, PI4III kinase α, delocalizes PH-domain-based reporters from the plasma membrane, suggesting that its function in providing PI4P at the cell surface is evolutionarily conserved with yeast (Balla et al., 2005). A PIK1 homolog, PI4III kinase β, is also present in mammals but studies suggest that it functions primarily to produce PI4P in the Golgi, where it is used to regulate trafficking to the cell surface (Godi et al., 2004; Wong et al., 1997). To a great extent, however, the precise degree to which STT4 and PIK1 function has been conserved and apportioned among their mammalian orthologs remains unclear.

In yeast, suppressor of actin-1 (sac1) phosphatase has been shown to dephosphorylate PI4P to PI, and its loss results in an 8- to 10-fold increase in the levels of PI4P (Foti et al., 2001). Sac1 is localized to the Golgi and the Endoplasmic Reticulum and is highly conserved in its sequence between Drosophila, mice, and humans. In Drosophila, sac1 was first identified as a lethal mutation with embryonic defects showing a puckering phenotype similar to that seen upon an increase of Jun Kinase signaling (JNK) (Wei et al., 2003). Loss of sac1 function causes an increase in Jun Kinase activity during dorsal closure and results in ectopic expression of decapentaplegic (dpp), the signal for the TGF-β pathway.

In this article, we show that loss of sac1 function causes ectopic activation of Hedgehog signaling. Our studies further show that this increased signaling upon loss of sac1 is due to PI4P accumulation and occurs at the level of Ptc and Smo interaction. Consistent with this observation, our results show that loss of sac1 and the gene encoding the kinase required for PI4P production generate Hh gain and loss of function phenotype, respectively, in multiple settings during Drosophila development. We propose a model in which control of lipid metabolism by Ptc plays a novel and critical role in transducing the Hh pathway signal.

RESULTS

Activation of Jun Kinase, Wg, and Dpp in sac1 Mutant Clones During Imaginal Disc Development

The previously studied function of Drosophila sac1 relates to its role in the activation of JNK during embryonic dorsal closure (Wei et al., 2003). Consistent with these studies, sac1 mutant clones generated in eye imaginal discs also show increased JNK signaling exemplified by a significant increase in the level of pJUN staining (Figure 1A), as well as by the increased expression of a JNK reporter gene (Figures 1B and 1C). Activation of the JUN kinase pathway in imaginal discs has been shown to cause cell death mediated by activation of Caspase-3 (Igaki et al., 2002). Indeed, Caspase-3 is activated in sac1 mutant clones (Figure 1D), and the resulting apoptotic signal is suppressed by mutations in the genes encoding JNK (basket [bsk]) and in the gene encoding the upstream kinase that activates the JNKKKK (missapen [msn]) (Figures 1E and 1F). Overexpression of such antiapoptotic genes as DIAP-1 also blocks cell death in sac1 mutant tissue (Figure 1G). This causally links Caspase activation in sac1 clones to the activation of the JNK pathway. A second major target of JNK signaling is wingless (wg) (Ryoo et al., 2004), and we found that Wg protein expression is also increased in sac1 mutant clones and that this ectopic Wg expression is also suppressed in msn/msn, sac1/ sac1 double mutant clones (Figures 1H and 1I).

In addition to these phenotypes, which largely confirm previous findings at other developmental stages where sac1 function was studied, we found that sac1 mutant clones also show a dramatic increase in the expression of the gene encoding the BMP ortholog in Drosophila, decapentaplegic (dpp) (Figures 2A–2B). Unlike Wg and Caspase-3, increased dpp expression is independent of JNK signaling because it is maintained in msn, sac1 double mutant clones (Figures 2Cand 2C’). Because dpp is a target of Hedgehog (Hh) in the eye imaginal disc, we investigated whether sac1 is associated with increased output from Hh signaling.

In the third instar eye disc, Ci, the transcriptional effector of Hh signaling, is activated in a narrow stripe of cells immediately anterior to the morphogenetic furrow that marks the advancing front of the differentiation wave (Figure 2D). In sac1 mutant clones, expression of activated Ci is dramatically increased both ahead and behind the furrow (Figures 2E and 2E’) and is...
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Figure 1. Activation of Jun Kinase Cascade and Caspase-3 in sac1 Mutant Clones
All tissues are third instar eye discs, posterior is to the left.

(A) In a disc containing sac1<sup>12F</sup>/ sac1<sup>12F</sup> mutant clones, anti-phospho-JNK staining (red) is upregulated in mutant tissue (nongreen).

(B) Control, mock clones in which both green and nongreen tissue are wild-type show normal weak wild-type expression of msn-lacZ reporter (red) in cells posterior to the furrow (marked by arrowhead).

(C) In a disc containing sac1<sup>12F</sup>/ sac1<sup>12F</sup> mutant clones (nongreen), msn-lacZ reporter (red) is overexpressed in the mutant tissue.

(D) In a disc containing sac1<sup>12F</sup>/ sac1<sup>12F</sup> mutant clone (nongreen), Caspase-3 (red) is activated in the mutant tissue.

(E) In a disc containing double mutant sac1<sup>12F</sup>/ sac1<sup>12F</sup> and bsk<sup>1</sup> bs[k<sup>1</sup> clones (noncolored) sac1<sup>2F</sup> clones with a single copy loss of bsk (blue) and wild-type cells (green), Caspase-3 (red) expression is fully suppressed in cells doubly mutant for sac1/bsk and partially suppressed in sac1-only mutant clones (blue), which are heterozygous for bsk (compare with D). bsk<sup>1</sup> is a null allele of JUN kinase in Drosophila.

(F) In a disc containing double mutant sac1<sup>12F</sup>, msn<sup>172</sup>/ sac1<sup>12F</sup>, msn<sup>172</sup> clones (nongreen), Caspase-3 (red) expression is suppressed in the mutant tissue (compare with D).

(G) DIAP1 was overexpressed using the Ay-Gal4 system (see Experimental Procedures) in a disc containing sac1 mutant clones (non-green). Cells mutant for sac1<sup>12F</sup>/ sac1<sup>12F</sup>, which also overexpress DIAP1, show a reduction in Caspase-3 (red) activation (compare with D).

(H) Wg (red) is overexpressed in mutant tissue in a disc containing sac1<sup>12F</sup>/ sac1<sup>12F</sup> mutant clones (nongreen).

(I) Wg (red) expression is suppressed in the mutant tissue in a disc containing sac1<sup>12F</sup>/ sac1<sup>12F</sup>, msn<sup>172</sup>/ msn<sup>172</sup> double mutant clones (nongreen) (compare with H).

independent of JNK signaling because it is maintained in msn, sac1 double mutant clones (Figures 2F and 2F). Additionally, loss of sac1 function causes high levels of membrane localized Smo to accumulate in the mutant tissue (Figures 2G–2H), and this phenotype is also independent of JNK signaling because it is maintained in msn, sac1 double mutant clones (Figures 2I and 2I). Ptc is a downstream target of Hh signaling and is also expressed at higher levels in sac1 mutant clones (Figures 2J–2K), and its expression is also maintained in sac1, msn double mutant combination (Figures 2L and 2L). Thus, transducers of Hh pathway and its downstream targets dpp and ptc are ectopically activated in sac1 mutant tissue in a JNK-independent manner. Furthermore, membrane-associated receptors and ligands of other signaling pathways, such as Notch and Delta, whose localization and trafficking has been reported to require PI function (Skwark and Boulianne, 2009), show no increase upon loss of sac1 function (see Figures S1A–S1F available online). Likewise, localization of proteins associated with plasma membrane, such as Crumbs, PDGF/VEGF receptor (PVR), and Armadillo, is unaffected in sac1 mutant clones (Figure S1G–S1O). These observations suggest that the loss of sac1 function does not result in general defects in protein transport to the membranes but specifically increases output from Hh signaling.

Interestingly, the expression of Hh protein remains unaltered in sac1 mutant clones (Figures 3A and 3A). Also, accumulation of ectopic Smo in sac1 is not altered in hh<sup>ts</sup> genetic background under conditions nonpermissive for hh<sup>ts</sup> function (Figures 3D and 3D). Thus, the Hh pathway is activated in sac1 mutant tissue downstream of the ligand binding event.

Sac1 Is Required for the Membrane Localization of Smo
In wild-type cells, Smo is largely localized to vesicles and translocates to the plasma membrane upon activation by Hh (Denef et al., 2000; Jia et al., 2004; Zhu et al., 2003). We further investigated the membrane localization of Smo in a sac1 mutant background using the previously described Drosophila salivary gland model (Zhu et al., 2003). In this system, all components of Hh signaling cascade, except the Hh protein, are normally expressed. Ectopic expression of Hh in these cells using antp-Gal4 as a salivary-gland-specific driver causes membrane localization of Smo and activation of the pathway (Figures 3E and 3F). In the antp-Gal4 UAS-sacI<sup>RNAi</sup> combination, where sac1 function is attenuated in the salivary gland, Smo is relocated to

the cell membrane even in the absence of Hh (Figure 3G).
Furthermore, this membrane localization of Smo promoted by
the loss of sac1 alone is sufficient to promote both the membrane
localization of Smo and its ability to activate downstream compo-
nents of the pathway.
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Increased Levels of PI4P in sac1 Mutant Tissue

Biochemical analysis in *Saccharomyces cerevisiae* has established that *sac1* encodes a lipid phosphatase whose primary substrate is PI4P (Foti et al., 2001). We used antibodies against PI4P to detect this lipid in eye discs containing *sac1* clones and found that the mutant tissue shows a dramatic increase in PI4P levels as compared to adjacent wild-type cells (Figures 4A and 4A'). This increase in the levels of PI4P upon loss of *sac1* function is not due to perturbations in the levels of other PIs, such as PI(4,5)P and PI(3)P, because their levels in *sac1* mutant tissue is similar to that seen in adjacent wild-type tissue (not shown). The synthesis and interconversion of PIs in cells is regulated by a balance in the activities of lipid phosphatases and kinases. When we used RNAi to down-regulate the STT4 kinase in *sac1* clones in the eye disc, PI4P levels were significantly reduced (Figures 4B and 4B'). Likewise, in the wing imaginal disc, a reduction of *sac1* function in the dorsal compartment of the wing disc using ap-Gal4, *UAS-sac1RNAi* combination resulted in an increase in PI4P levels (Figures 4C and 4C'); a simultaneous loss of STT4 kinase in this genetic background also caused a significant reduction in PI4P levels (Figures 4D and 4D'). In contrast, a simultaneous loss of *four wheel drive* (fwd), the ortholog of yeast PLK1 (Brill et al., 2000), in *fwd*, *sac1* double mutant clones in the eye disc and coexpression of *UAS-sac1RNAi* and *UAS-fwdRNAi* in the dorsal compartment of the wing disc do not result in suppression of PI4P phenotype of *sac1* (Figure S2). This finding establishes *Drosophila* STT4 as a major kinase required for PI4P generation in the context of Hh signaling. To further determine the epistatic relationship between the Sac1 phosphatase/STT4 kinase pair and the canonical components of Hh signaling, we used the wing disc as a model because appropriate region-specific drivers are available for this tissue.
In the third instar wing imaginal disc, Hh is expressed in cells of the posterior compartment and activates the signaling cascade in the anterior compartment in a graded fashion (Figure 5A). Ci, the transcriptional effector of Hh signaling, is expressed in all cells of the anterior compartment, whereas the transcription of Dpp, transcriptional effector of Hh signaling, is expressed in all cells of the anterior compartment in a graded fashion (Figure 5A). Ci, the posterior compartment and activates the signaling cascade in the posterior half of the dorsal compartment (Figures 5D and 5D'). These results strongly argue for a requirement of the STT4 kinase that gives rise to PI4P in the dorsal compartment of the wing disc (green) causes PI4P (red) levels to be significantly reduced as compared to loss of sac1 function alone (compare with C and C').

Finally, loss of STT4 function in the dorsal compartment of the wing disc causes a strong reduction in the expression of Ptc, a Hh target gene (Figures 5F–5G) and to a lesser extent the expression of activated Ci in the dorsal-anterior compartment indicating an attenuation of Hh signaling (Figures S3A and S3B).

The Ptc protein plays perhaps the most prominent role in the regulation of Smo membrane localization during Hh signaling (Jiang and Hui, 2008; Zhu et al., 2003). For example, overexpression of Ptc in the dorsal compartment of the wing disc using the genetic combination ap-gal4, UAS- ptc causes a loss of membrane associated Smo in the posterior half of the dorsal compartment (Figures 5H and 5H'). We show that overexpression of Ptc can also override the Smo membrane localization phenotype and PI4P accumulation seen as a result of down-regulation of sac1 (Figures 5I and 5I'; Figures S3C–S3F). Thus, ptc functions along with sac1 in the regulation of membrane localization of Smo.

In wild-type third instar wing disc, dpp-lacZ expression is dependent on high levels of Hh signaling and is seen as a stripe of cells in the anterior compartment along the A/P boundary (Figure 5B). This expression is expanded in the dorsal-anterior compartment when sac1 function is reduced in the dorsal compartment of the wing disc (Figures 5J and 5J') indicating increased Hh signaling in cells that normally respond to Hh. However, a simultaneous loss of STT4 and sac1 in the dorsal compartment of the wing disc prevents this expansion of dpp-lacZ expression and causes a suppression of the sac1 phenotype (Figures 5K and 5K'). In this combination, lower sac1 levels would increase PI4P levels, but the STT4 kinase is required for the generation of PI4P in the first place. Thus, the simultaneous block of STT4 and sac1 will cause a reduction in PI4P leading to...
Figure 5. *sac1* Phosphatase and *stt4* PI4 Kinase Cause Elevated Membrane Levels of Smoothened Protein

The wing pouch is marked by a dotted line. Red channel only shown in gray scale in (C), (D'), (E'), and (G') for clarity.

(A) A schematic representation of the third instar wing disc. The wing pouch is marked by white dotted line. The A/P compartment boundary (A/P) demarcates the anterior and posterior compartments. The D/V compartment (D/V) boundary demarcates the dorsal and the ventral compartments. *ap-Gal4* is expressed throughout the dorsal compartment (shown in blue). Smo is expressed in the posterior compartment of the pouch (red dots).

(B) In wild-type, Smo (red) is expressed in the posterior compartment, whereas the Hh target reporter *dpp-lacZ* (green) is activated along the A/P compartment boundary.

(C and C') Knockdown of *sac1* in the dorsal compartment of the wing disc using the combination *ap-Gal4, UAS-GFP; UAS-sac1RNAi* (green cells), causes Smo (red) membrane localization to expand to the dorsal-anterior compartment of the wing (arrow) (compare with B). Red channel only shown in gray scale for clarity (C').

(D and D') Knockdown of *stt4* kinase in the dorsal compartment of the wing disc using the combination *ap-Gal4, UAS-stt4RNAi UAS-GFP* (green) is shown. Smo (red) membrane localization is reduced in the dorsal posterior compartment (arrow). Red channel is only shown in gray scale for clarity (D').

(E and E') Double knockdown of *sac1* and *stt4* in the dorsal compartment of the wing disc using *ap-Gal4, UAS-stt4RNAi; UAS-sac1RNAi* (green) genetic combinations is shown. Smo (red) accumulation in the dorsal anterior compartment (arrow) and dorsal posterior compartment (arrowhead) is significantly reduced. Red channel is only shown in gray scale for clarity (E').

(F and F') As a control, wild-type Ptc (red) is expressed along the A/P boundary at similar levels in cells of the dorsal (green) and ventral (nongreen) regions of the wing pouch.

(G and G') Knockdown of *stt4* kinase in the dorsal (green) compartment of the wing disc using the genetic combination *ap-Gal4, UAS-stt4RNAi UAS-GFP* causes significant reduction in Ptc (red) expression.

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a reduction in dpp-lacZ expression, a readout for Hh signaling. These results link phospholipid metabolism with the ability of Smo to signal and activate Hh signaling. To directly test whether PI4P levels increase upon activation of Hh signaling, we once again used the salivary gland as a model. In wild-type, salivary gland cells contain low PI4P that increases dramatically when gland cells contain low PI4P that increases dramatically when sac1 is down-regulated (Figures 6A–6A’). Likewise, activation of Hh signaling by removing ptc in flip-out clones using the Ay-gal4 system in the salivary gland (see Experimental Procedures) using the combination Ay-Gal4 UAS- ptcRNAi, which causes a loss of ptc expression in cells expressing Gal4 (marked by GFP) and causes a cell-autonomous increase in its PI4P content (Figures 6B–6B’). Similarly, an increase in PI4P content is seen when ptc function is attenuated in the dorsal compartment of the wing imaginal disc using the combination ap-gal4, UAS- ptcRNAi or when mutant clones of ptc are analyzed in the eye imaginal disc (Figures S3G and S3H). The increase in PI4P levels upon loss of ptc function is dependent on STT4 function because simultaneous loss of ptc and STT4 function in the combination ap-gal4, UAS- ptcRNAi and UAS-STT4RNAi results in the suppression of the phenotype (Figures S3I–S3L). This establishes a causal relationship between the strength of the Hh signal and the level PI4P. Additionally, the change in level of PI4P is not a feedback from a posttranscriptional target of Hh signaling because overexpression of activated Ci in the dorsal compartment does not result in an increase in PI4P levels, nor does a simultaneous loss of ptc and smo in the dorsal compartment of the wing disc using the combination ap-gal4, UAS- ptcRNAi, UAS-smoRNAi suppress the increased levels of PI4P (Figures S3M–S3P).

STT4 and Sac1 Are Conserved Components of the Hedgehog Signaling Pathway

Results presented thus far suggest that STT4 functions as a positive modulator of Hh signaling, whereas Sac1 is required to attenuate this signal. To test whether loss of STT4 and sac1 in the embryo results in the signature hedgehog segment polarity phenotypes, we microinjected hairpin versions of STT4 RNA (because no loss of function alleles are available) and for sac1, we isolated homozygous loss of function sac1^{L2F}/ sac1^{L2F} mutants and monitored their denticles belt phenotypes. In wild-type late stage embryos, there are 14 denticles belts with a distinct segmental polarity pattern (Figures 6C–6C’). As positive controls, microinjection of a short hairpin version of Smo RNA exhibits overspecification of denticle belts and disruption of polarity (Figures 6D and 6D’). Ptc mutant embryos show reduction in the denticle belts (Hooper and Scott, 1989), sac1^{L2F} homozygous mutant embryos show a reduction in the width of the denticle belts, a phenotype similar to that seen in ptc, indicating of increased Hh signaling (Figures 6E and 6E’), whereas loss of STT4 results in overspecification of denticle belts (Figures 6F and 6F’), a phenotype similar to that seen in hh and smo mutant embryos (compare Figures 6F and 6F’ with Figures 6D and 6D’), further establishing that STT4 functions as a positive modulator of Hh signaling. Because of the nature of feedback loop between Hh and Wg at the segmental boundary, this effect of sac1/STT4 on Hh is likely to trigger a change in Wg levels as well.

Many aspects of Hedgehog signal transduction, though not all, are evolutionarily conserved between Drosophila and mammals. We explored the possibility that PI4P signaling modulates Hh signal transduction in mammalian cells. The mouse genome encodes an ortholog of STT4, called PIKIII kinase z, as established by domain architecture and catalytic domain primary sequence alignment. A second mouse kinase, PIKIII kinase b, has a domain architecture that appears to be more closely related to PIK1 (Balla and Balla, 2006). The effect of kinase subunit RNA interference was tested in Shh-LIGHT2 cells, a mouse fibroblast cell line engineered to possess a Gli-dependent luciferase-based reporter system (Taitale et al., 2000). Treatment of these cells with Shh gives robust (>10-fold) increases in reporter activity. In a control experiment, Shh-LIGHT2 cells were treated with RNAi that inactivates smo RNA. Loss of Smo function reduced Shh-induced reporter activation, demonstrating the effectiveness of this system in measuring effects on Hh pathway transduction (Figure 6G). Next, we tested RNAi treatments designed to reduce kinase functions. RNAi against PI3 kinase or PI4II kinases did not affect Shh-driven reporter induction to a significant extent. In contrast RNAi against the mammalian STT4 homolog, PIKIII kinase b, strongly reduced Shh-stimulated reporter activity (Figure 6G). RNAi against the mammalian PIK1 homolog, PI4III kinase b, also affected reporter induction in these cells. The RNAi sequences used were each highly specific for the kinase target, so the results are unlikely to be explained by cross-reactivity. Taken together, these results suggest a nonredundant role for PI4III kinases in mammalian Hh pathway transduction. Note that RNAi against the mammalian ortholog of Sac1 did not affect reporter activity, either in basal or Shh-stimulated conditions (data not shown). However, Shh-LIGHT2 cells are relatively insensitive to Hh pathway derepression caused by the removal of negative regulators such as Suppressor of Fused (data not shown).

DISCUSSION

A major regulatory step in the modulation of Hedgehog signaling occurs at the level of the two multipass transmembrane proteins, Patched and Smoothered. Genetic and biochemical studies
suggest that the ligand Hh binds Ptc and functions in its inactivation (Taipale et al., 2002; Denef et al., 2000). This inhibitory step is critical for the activation of Smo, which transduces the signal intracellularly to promote Hh target gene activation. The importance of this regulatory step is further underscored by the observation that the Ptc/Smo interaction is the most commonly disrupted step in cancers caused upon aberrant Hh signaling (Rohatgi and Scott, 2007).

Figure 6. STT4 Kinase and Sac1 Phosphatase Function in Hh Signaling
(A and A′) Loss of sac1 function in positively marked flip-out clones (A, green) generated using the Ay-Gal4 UAS-sac1 RNAi combination in salivary gland shows elevated levels of PI4P (A′, red). The merged panel is shown in (A′).
(B and B′) Loss of ptc function in positively marked flip-out clones (B, green) generated using the Ay-Gal4, UAS-ptc RNAi combination in salivary gland shows elevated levels of PI4P (B′, red). The merged panel is shown in B′. The outline of the salivary gland and wild-type cells is marked with white dotted line.
(C and C′) Organization of denticle belts in wild-type Drosophila embryo microinjected with buffer. A higher magnification of panel C′ emphasizes normal polarity of denticle belts.
(D and D′) Denticle belt preparation from embryo microinjected with smod dsRNAi is shown. The denticle belt pattern is disrupted with the broadening of each belt and a loss in its polarity (magnified image in D′).
(E and E′) sac1 2F homozygous embryo showing reduction in denticle belt specification, a phenotype similar to that seen in weak ptc mutant embryos.
(F and F′) Denticle preparation of microinjected embryo with STT4 dsRNAi shows expansion of denticle belt specification similar to that seen in smod dsRNAi (compare with D and D′).
(G) PI4III kinase RNAi inhibits Hh pathway activation in mammalian cells. Diced siRNA pools were made against PI4III kinase α and PI4III kinase β, the mammalian STT4 and PIK1 homologs, respectively. RNAi against other PI4 kinases and PI3 kinase were also tested, along with RNAi against Smo. Hh reporter cells were treated with the indicated RNAi, then grown to confluency and switched into low-serum growth medium containing Shh. Following 24 hr Shh treatment, cells were lysed and assayed for firefly luciferase-based reporter induction relative to a constitutive Renilla luciferase. Data are reported as the mean of three replicates ± one SD. *p < 0.01, Student’s t test (two-tailed).
(H) The genetic results presented in this model are consistent with a model in which Ptc inhibits PI4P formation, and this causes retention of Smo away from the plasma membrane. Hh binding to Ptc would activate STT4 kinase and the increase of PI4P will cause a nonstoichiometric amount of Smo to be transported to the cell membrane.
In this article, we show that phospholipid metabolism plays an important role in the modulation of Hh signaling at the level of Ptc/Smo interaction. In particular, our results show that an increase in the level of PI4P by the inactivation of Sac1 phosphatase leads to Smo protein relocalization to the membrane and an increase in Hh signaling in multiple tissues during Drosophila development. Furthermore the kinase (STT4), which is required for the generation of PI4P, is also required for the proper transduction of Hh signaling as indicated by its effects on Hh target gene expression. PI4P accumulation in the cell is a hallmark of sac1 mutations and is also seen upon loss of ptc activity. Furthermore, in sac1 mutant tissue, we find both increased membrane localization of Smo and accumulation of PI4P, whereas reduction in the PI4P kinase function leads to an hh-like loss of function phenotype. These results establish that phospholipid metabolism provides a critical regulatory input in the modulation of Hh signaling.

Recent studies have proposed that Smo activation requires an input from a nonprotein small molecule. Cholesterol and its derivatives (oxysterols) are likely candidates for the small molecules required directly or indirectly for Ptc inhibition or Smo activation, because they also promote the translocation of Smo to the cilium (Dwyer et al., 2007). Because oxysterols are known to bind to vesicular transport proteins that also interact with phospholipids (Xu et al., 2001), further studies on possible cooperation between these two lipid types could further shed light on the mechanism of Smo activation.

Inactivation of Smo by Ptc occurs in a catalytic fashion (Taitale et al., 2002) in that a small number of Ptc molecules can inactivate many more Smo molecules. Our results provide an explanation for this nonstoichiometric inhibitory mechanism. The finding that inactivation of Ptc increases PI4P suggests that Ptc normally functions in keeping PI4P levels low within a cell. This could be achieved either by the down-regulation of the STT4 kinase or by the up-regulation of the Sac1 phosphatase. It is less likely that Ptc modulates Sac1 activity because studies in multiple model systems have shown that Sac1 is predominantly localized to the Golgi and, as a result of proximity arguments alone, it seems a more likely possibility that Ptc modulates PI4P levels by down-regulating the lipid kinase. In this model, during normal Hh signaling, binding of Hh to Ptc will relieve repression of the kinase by Ptc and cause an increase in PI4P. As with all genetic analysis in Drosophila, our results do not imply direct protein interactions; currently unknown transduction components could exist, and future biochemical analyses will reveal which, if any, of the interactions is direct. However, our genetic analysis does allow us to propose how an increase in the levels of this lipid can activate Hh signaling. Studies from both flies and vertebrate model system have suggested that the localization of Smo protein to the plasma membrane is essential for the activation of the pathway, and studies in multiple model systems have shown that PI4P function is essential in the vesicular transport of cargo proteins from the Golgi to the plasma membrane (Skwarek and Boulianne, 2009). We therefore propose that Hh binding to Ptc releases inhibition of a lipid kinase such as STT4, resulting in high PI4P levels. This aids vesicular transport of Smo to the membrane and causes its activation. A schematic representing the genetic model that is consistent with past and present data is shown in Figure 6H. Our results using Shh-responsive mouse fibroblasts indicate that mammalian Hh signal transduction is dependent on the activity of the murine STT4 ortholog, PI4III kinase z. Previous localization studies suggest PI4III kinase z contributes to plasma membrane PI4P pools, an observation consistent with a conserved role for PI4P metabolites in the control of Smo by mammalian Ptc1 (Balla et al., 2005; Wong et al., 1997). The observation that RNAi against the mammalian PIK1 homolog, PI4III kinase b, also reduces Hh signal transduction could suggest it has diverged in function between flies and mammals. Alternatively, PI4P pools could be exchanged more readily between membrane-bound subcellular compartments and the cell surface in mammalian cells, making the removal of either of the PI4III kinases affect global availability of PI4P derivatives. In mammalian cells, Smo activation is associated with translocation of the molecule to the primary cilium, a ubiquitous microtubule-based cell surface protrusion (Corbit et al., 2005; Huangfu et al., 2003). Given that Drosophila cells appear to lack primary cilia, it will be of interest to determine whether PI4III kinase activity is required for Smo translocation.

**Experimental Procedures**

**Fly Stocks**

The following stocks were used in this study: FRT40A and FRT80B (Bloomington), w; msn725 P(neoFRT)180B/TM6B (J. Treisman), yw; msn1/Y2/TM3, Sb (Y. N. Jan), sac1^+ (H. Weil), sac1^a1;g222X2 (Bloomington), bsk1^+/Oyo (M. Seeger), UAS-DIAP1 (B. Hay), Pk61CFLP37 (Szeged), hh^{w1} (K. Moses), dop-lacz (Bloomington), UAS-sac1^RNAi (VDRC 37217), UAS-stds^RNAi (VDRC 15993), UAS-fwe^RNAi (VDRC 27785), fww7^yos37 (Bloomington), puc-lacz (Bloomington), UAS-pc{fl}^RNAi (NIG-FLY 2411R-1), UAS-smo^RNAi (NIG-FLY 1156R-1), UAS-smo^yfp, UAS-cl (K. Basler), UAS-hh (P. Ingham), and Ay-Gal4 (Bloomington).

**Immunohistochemistry and Embryonic ds-RNA Injections**

We used the following antibodies: mouse anti-b-galactosidase (1:100; Promega); rat anti-Ci (1:50; R. Holmgren); rabbit anti-cleaved caspase-3 (1:200; Cell Signaling); rabbit anti-Hh (1:1,000; Ingham, P.); mouse anti-Ptc and mouse anti-Smo, anti-Crumbs, anti-Dl, and anti-Notch (1:50; 1:20, 1:100, 1:20); Hybridoma Bank, Iowa); mouse anti-PI4P (1:100; Echelon); mouse anti-Wg (1:100; Hybridoma); and rabbit anti-phospho JNK (1:50; Cell Signaling). Antibody staining were performed as described elsewhere (Rogge et al., 1995), except that anti-PI4P staining (Blagoveshchenskaya et al., 2002) was performed using 3% Saponin in 1X TBS (Sigma S7900) during washes and antibody incubation. ds-RNA and denticle preparation experiments were performed as described elsewhere (Kennerdell and Carthew, 2000).

**Ay-Gal4 Flip-Out Clones**

Flip-out clones were generated in the eye and in the salivary gland. In the eye discs, ey-flp was used to flip out the stuffer cassette from act-FRTyFRT-Gal4 to generate act-FRTGl4, which expresses Gal4 in all cells of the eye imaginal disc (Ito et al., 1997). In the salivary gland, hs-flp was used as a source for flp. The respective crosses were maintained at 18 C; when the larvae reached midsecond instar, a brief heat shock (10 min) was given at 37 C, and when the larvae reached third instar, salivary glands were dissected out, fixed, and stained with appropriate antibodies.

**Imaging**

Samples were imaged using a BioRad Radiance 2000 confocal with Laser-Sharp 2000 acquisition software. Fluorescent intensity quantifications were analyzed by use of ImageJ software.

**Shh Reporter Assay**

Diced siRNA pools were generated using previously described methods (Myers et al., 2003). Gene-specific PCR primers were used to amplify...
~550 bp segments from the coding region of each of the gene indicated in Table S1. A second round of PCR was used to attach forward and reverse T7 polymerase binding sites to the gene-specific PCR products. This DNA was used as template for the production of long dsRNA using in vitro transcription (Ambion). The dsRNA products were processed into 21-bp fragments using recombinant RNaseI enzyme, resulting in diced siRNA pools directed against each target gene (NEB).

To conduct mammalian Hh pathway reporter assays, diced siRNA pools were introduced into Shh-LIGHT2 cells using a reverse transfection procedure. For each well of a 96-well plate, Lipofectamine 2000 (Invitrogen) was complexed with 10 pmol of siRNAs in 50 μl of Opti-MEM (GIBCO) in individual wells. Early-passage Shh-LIGHT2 cells in log-phase growth were trypsinized, counted, and adjusted to a concentration of 200 cells/μl. One hundred microliters of culture medium containing 2 x 10^4 cells was gently added to each well, atop the transfection mixture. One day after transfection, the culture medium was changed to DMEM containing 0.5% FBS with or without the addition of Shh-conditioned medium. After 24 hr incubation with Shh-conditioned medium, cells were lysed and luciferase signals were read using the Dual Luciferase Reporter Assay System (Promega). Data are reported as ratios of Hh-dependent firefly luciferase signal to constitutive Renilla luciferase signal.

**SUPPLEMENTAL INFORMATION**

Supplemental information includes three figures and one table and can be found with this article online at doi:10.1016/j.devcel.2010.06.007.

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