Smoothened regulation in response to Hedgehog stimulation

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Abstract

The Hedgehog (Hh) signaling pathway play critical roles in embryonic development and adult tissue homeostasis. A critical step in Hh signal transduction is how Hh receptor Patched (Ptc) inhibits the atypical G protein-coupled receptor Smoothened (Smo) in the absence of Hh and how this inhibition is release by Hh stimulation. It is unlikely that Ptc inhibits Smo by direct interaction. Here we discuss how Hh regulates the phosphorylation and ubiquitination of Smo, leading to cell surface and ciliary accumulation of Smo in *Drosophila* and vertebrate cells, respectively. In addition, we discuss how PI(4)P phospholipid acts in between Ptc and Smo to regulate Smo phosphorylation and activation in response to Hh stimulation.

Keywords

Hh signaling; phospholipid; phosphorylation; Smo; ubiquitination; signal transduction

Hh signaling

Hedgehog (*hh*) was first discovered as a segment polarity gene involved in *Drosophila* embryo development (Nüsslein-Volhard and Wieschaus, 1980). The Hh signaling pathway controls the most organs development and post developmental tissues homeostasis (Ingham and McMahon, 2001; Jiang and Hui, 2008; Briscoe and Thérond, 2013), malfunction of which causes birth defects as well as several types of cancer, including medulloblastoma, basal cell carcinoma (BCC), breast cancer, and lung cancer, thereby it has become an attractive therapeutic target.

The Hh signaling is transduced by a signaling cascade that is highly conserved among species, with the receptor complex consisting of two membrane protein, a 12-span transmembrane protein Patched (Ptc) and a seven-span transmembrane protein Smoothened (Smo). In the absence of Hh, Ptc inhibits Smo activity by promoting Smo endocytosis and turnover in intracellular compartments (Denef et al., 2000). Ptc likely inhibits Smo catalytically (Taipale et al., 2002), because substochiometric levels of Ptc are able to repress Smo activation (Taipale et al., 2002; Casali and Struhl, 2004). In *Drosophila*, the Hh signal

is transduced through the receptor complex Ptc-Interference Hh (Ptc-Ihog) and the signal transducer Smo at the plasma membrane (Lum and Beachy, 2004; Camp et al., 2010; Zheng et al., 2010). Binding of Hh to Ptc-Ihog relieves the Ptc-mediated inhibition of Smo, which allows Smo to activate the Cubitus interruptus (Ci)/Gli family of zinc finger transcription factors and thereby induce the expression of Hh target genes, such as *decapentaplegic* (*dpp*), *ptc, collier* (*col*) and *engrailed* (*en*) (Hooper and Scott, 2005; Jia and Jiang, 2006) (Fig. 1A). Unlike *Drosophila* has single Ptc, Hh, and Gli genes, mammals have two Ptc receptors-Ptc1 and Ptc2, three Hh ligands-Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh), and three Gli transcription factors-Gli1, Gli2, and Gli3 (Jia and Jiang, 2006).

Smo, an atypical G protein-coupled receptor (GPCR), is the key positive regulator of the pathway in both insects and vertebrates. Abnormal activation of Smo results in several cancers, thus Smo is an attractive therapeutic target (Xie et al., 1998; Yang et al., 2010). Currently, the most advanced drug is vismodegib that was approved by Food and Drug Administration (FDA) for treating cancers driven by Smo activation (Sekulic et al., 2012; Tang et al., 2012). However, cancer cells can acquire resistance and vismodegib becomes ineffective (Atwood et al., 2015; Sharpe et al., 2015). Several different mechanisms of resistance have been reported, including those through mutations in Smo (Wang et al., 2013), activation of Gli2 (Buonamici et al., 2010), mutations in the negative regulator Suppressor of fused (Sufu) (Kool et al., 2014), and activation of RAS/MAPK pathway (Zhao et al., 2015). Therefore, a better understanding the mechanisms of Smo regulation in the fundamental development processes is critical to developing more effective therapeutic treatments for cancers caused by Smo dysregulation.

Primary cilium is a microtubule-based short membrane protrusion found in most mammalian cells, and primary cilium dysfunction is the basis of a series of human pathologies, such as cancer, cystic kidney, obesity, blindness, cognitive disabilities, mental retardation, and various developmental malformations (Goetz and Anderson, 2010). In mammals, most of the Hh signaling components are located at or nearby the primary cilium, which is an absolute requirement for proper Hh signaling (Briscoe and Thérond, 2013). In the absence of Hh, Ptc1 is concentrated in the primary cilium, while in the presence of Hh Ptc1 is removed from the cilium, whereas Smo accumulates in the cilium that is required for its activation (Corbit et al., 2005; Rohatgi et al., 2007; Rohatgi et al., 2009; Wang et al., 2009) (Fig. 1B). Kim et al. identified that Ptc contains the ciliary localization sequence in the C-terminal domain, which is necessary to mediate protein localization to the primary cilium (Kim et al., 2015). Surprisingly, Ptc must accumulate in the cilium to inhibit Smo in the absence of Hh, but Ptc ciliary removal is secondary because Ptc mutants that are unable to exit the cilium not only repress Smo activity but also enabled Hh to neutralize its repression and elevate Smo activity in the presence of Hh (Kim et al., 2015). Increased ciliary accumulation of Smo is associated with activation of Gli transcription factors and expression of Hh target genes. Gli proteins are the transcriptional effectors of Hh pathway, the balance between activator and repressor forms of Gli2 and Gli3 is critical to the activity of the pathway. In addition, the base of the cilium acts as a gate to the basal body, which mediates the exchange of Hh signaling components in and out of the cilium. The cAMP-activated kinase, PKA,

functions at the base of the cilium and is key in negative regulating Hh signaling by mediating the processing of Gli into Gli^R (Jiang and Struhl, 1998; Wang et al., 2000a; Tuson et al., 2011). Therefore, cilium is fundamentally function as a signaling center for the Hh pathway in mammals (Wilson and Chuang, 2010). However, it remains unclear how Smo cell surface/ciliary accumulation and intracellular trafficking are controlled.

Two products of human disease genes responsible for the Ellis-van Creveld syndrome, Evc and Evc2, are reported to have the positive role in Hh signaling (Dorn et al., 2012; Yang et al., 2012; Pusapati et al., 2014). The loss of Evc/Evc2 does not affect Shh-induced Smo phosphorylation and ciliary localization but blocks Hh pathway activation mediated by constitutively active forms of Smo, suggesting that Evc and Evc2 are recruited to activate Gli proteins by antagonizing Sufu in the primary cilia (Yang et al., 2012). Evc/Evc2 are optional for the constitutive Gli activity in Sufu (−/−) cells, suggesting that Evc/Evc2 act upstream of Sufu to promote Gli activation. In addition the EFCAB7-IQCE complex anchors the EVC-EVC2 at the base of cilium, and Smo-Evc signaling complex is required for Hh signal transmission (Dorn et al., 2012; Pusapati et al., 2014). Primary cilium also serve as specialized compartments for calcium signaling, containing a heteromeric PKD1L1-PKD2L1 channel in mice and humans, which regulates ciliary calcium concentration thereby regulates ciliary Smo-mediated Gli1 expression and Gli2 translocation (DeCaen et al., 2013; Delling et al., 2013). Further studies could determine whether other components in the Hh pathway are calcium signaling dependent, and respond to changes in the environment during development.

Drosophila cells do not have cilium during development, hence cilium-mediated Hh signaling is restricted to vertebrates. Kuzhandaivel et al. identifed and characterized a ciliamediated Hh pathway in *Drosophila* olfactory sensory neurons (Kuzhandaivel et al., 2014). They demonstrated that Costal2 (Cos2) and Fused (Fu) are required for the Smo ciliary transport and cilium mediate the expression of the Hh signaling target genes. The ciliary Hh pathway in olfactory sensory neurons is conserved, at least in part, from *Drosophila* to vertebrates.

Smo phosphorylation regulated by different levels of Hh signaling activity

Studies of the Hh signaling in the past have identified many core components that control the Hh signaling pathway (Wilson and Chuang, 2010), most of which are regulated by phosphorylation (Chen and Jiang, 2013; Jia, 2012). Smo has homologyto GPCRs (Alcedo et al., 1996), and Smo signals need Gαi, one of the G-proteins in *Drosophila*, to activate low threshold of Hh target genes expression (Ogden et al., 2008). Like many GPCRs, phosphorylation of Smo controls the switch between on and off of the signaling states.

In *Drosophila*, studies have shown that Hh induces cell surface accumulation and phosphorylation of Smo by multiple kinases, including protein kinase A (PKA) and casein kinase 1 (CK1) (Jia et al., 2004; Zhang et al., 2004; Apionishev et al., 2005), casein kinase 2 (CK2) (Jia et al., 2010), G protein-coupled receptor kinase 2 (Gprk2) (Chen et al., 2010) and atypical PKC (aPKC) (Jiang et al., 2014), which activate Smo by inducing differential phosphorylation, thus the gradual conformational change in the protein (Jia et al., 2004;

Jiang et al., 2014; Apionishev et al., 2005; Zhang et al., 2004; Zhao et al., 2007; Chen et al., 2010; Jia et al., 2010; Fan et al., 2012;).

Smo forms homodimer and undergoes a conformational change when it is phosphorylated by PKA and CK1 upon Hh stimulation (Zeng et al., 2005; Zhao et al., 2007). Zhao et al. using fluorescence resonance energy transfer (FRET) analysis, propose a dimerization model to explain that Smo conformational states rely on the level of Hh activity and Smo phosphorylation, which can be manipulated by substitution of various Ser/Thr residues into either Ala or Asp (Zhao et al., 2007). PKA and CK1 play an essential role in activating Smobyphosphorylating Smoat three clusters of serine residues in its C-tail (Zhao et al., 2007; Aikin et al., 2008), but it would be interesting to understand how phosphorylation of Smo at the three clusters of residues contributes to its activity. Fan et al. demonstrated that Smo is differentially phosphorylated in response to different levels of Hh signaling activity (Fan et al., 2012). They also found in addition to the number of phosphorylated sites, the position of the phosphorylated residues is critical for establishing the stoichiometry of Smo activity. Thereupon a zipper-lock model, in which phosphorylation at the three clusters of serine residues promotes a gradual conformational change to activate Smo, is proposed (Fan et al., 2012).

Smo phosphorylation has been reviewed elsewhere (Jia, 2012; Chen and Jiang, 2013), here we only discuss the roles of atypical protein kinase C (aPKC) in regulating Smo phosphorylation. Evidence shows that Shh increases the PKC activity and Shh-induced increase of Gli1 mRNA level requires PKC in mouse embryonic stem cells (Heo et al., 2007), indicate that PKC may play role in Hh signaling pathway. Jiang et al. indicated aPKC has a positive role in regulating Hh signaling by modulating both Smo and Ci (Jiang et al., 2014). Smo phosphorylation at Ser680 by aPKC promotes its activation, while Ci phosphorylation at Thr512 and Thr590 by aPKC promotes the DNA binding activity of Ci Zn finger DNA binding doma in. These findings indicate that aPKC is directly involved in Hh signaling beyond its roles in cell polarity regulation. In addition, inhibition of aPKCι/λ suppresses the Hh signaling and the growth of BCC cell lines, and aPKCι/λ phosphorylates Gli1 to regulate its DNA binding activity (Atwood et al., 2013). Interestingly, Hh signaling upregulates aPKC expression in a Ci dependent manner (Jiang et al., 2014), while aPKC-ι/λ is upregulated in BCCs, and *Prkci* is a direct target gene of Gli1(Atwood et al., 2013). These studies uncover a conserved role of aPKC in upregulating Hh signaling activity and a positive feedback regulation of aPKC itself. Moreover aPKC form a complex with Par6 to regulate the activity of Smo and Ci, which is independent of their function in apical/basal polarity, because inactivation of several other polarityproteins, such as Crb, Baz, Dlg5, Dlg1, and Lgl, shows polarity phenotypes but does not affect Smo accumulation and Hh signaling in *Drosophila* wings (Jiang et al., 2014). It is unclear whether these polarity proteins have additional roles in Hh signaling through polarity regulation.

Phosphorylation mediates the balance between transcriptional activator and repressor forms of Ci/Gli, which plays dual roles by two distinct forms (Lum and Beachy, 2004; Jia and Jiang, 2006). In the absence of Hh, full-length Ci (Ci^F) undergoes proteolytic processing to generate a truncated repressor form (Ci^R), which functions as a repressor to block the expression of Hh responsive genes such as *dpp* (Hooper and Scott, 2005; Jia and Jiang,

2006). Proteasome-mediated Ci processing requires cooperative and sequential phosphorylation by PKA, glycogen synthase kinase 3 (GSK3) and CK1 at multiple serine/threonine (Ser/Thr) residues in the C-terminal region of Ci (Jiang and Struhl, 1995; Jia et al., 2002; Price and Kalderon, 2002). In *Drosophila*, similar to the phosphorylation clusters in Smo, PKA and CK1 phosphorylate Ci at three clusters in the absence of Hh to inhibit pathway activation, while in the presence of Hh, PKA and CK1 phosphorylate Smo to stimulate its signaling activity. So how different substrates are chosen by the same set of kinases in different signaling states is important to the field. Recently, two studies demonstrated that Hh promotes the accumulation of Smo by inducing a switch from the association of PKA-Ci-Cos2 to a PKA-Smo-Cos2 complex, leading to the Smo phosphorylation and Hh signaling activation (Li et al., 2014; Ranieri et al., 2014).

Cos2 mainly acts as a negative regulator in Hh signal transduction, as it inhibits the transcriptional activator activity of Ci^F by inhibiting its nuclear translocation (Sisson et al., 1997; Chen et al., 1999;Wang et al., 2000b). Cos2 also acts as a scaffold protein to bring PKA, CK1, and GSK3 to Ci^F, thereby promoting sequential phosphorylation of Ci^F by these kinases (Zhang et al., 2005; Price, 2006). Cos2 has a negative role on Smo, because it blocks the phosphorylation and activation of Smo likely by masking its phosphorylation sites (Liu et al., 2007). In addition, Cos2 has a positive role in Hh-responding cells, and this correlates to its ability to form a complex with the C-terminal intracellular tail of Smo (Wang et al., 2000b; Jia et al., 2003; Lum et al., 2003; Ho et al., 2005). Cos2 can be phosphorylated at Ser572 and Ser931 by Fused (Fu), which likely inhibits the negative activity of Cos2 on Smo phosphorylation and leads Cos2 distribution from the cytoplasm to the plasma membrane, which also elevates Ci nuclear translocation and transcriptional activity (Ranieri et al., 2012).

One important question is how Hh regulates Smo phosphorylation and cell surface accumulation. Although Smo phosphorylation promotes its cell surface accumulation and signaling activity, the mechanisms controlling its cell surface accumulation are still unclear. Smo physically interacts with the Cos2-Fu complex to transduce Hh signaling (Robbins et al., 1997; Jia et al., 2003; Lum et al., 2003; Ruel et al., 2003). Accumulation of Smo to the cell surface recruits the large protein complex, which is thought to induce dissociation of the Cos2-Ci-Kinase complex and hence inhibition of Ci processing (Zhang et al., 2005). Fu-Cos2 protein complex have feedback regulation on Smo, because Cos2-Smo interaction blocks Hh-induced Smo phosphorylation, and Fu is essential for Hh-induced Smo phosphorylation and cell surface accumulation by antagonizing Cos2 (Liu et al., 2007). Fu is in the non-phosphorylated inactive state in the absence of Hh and a phosphorylated active state in the presence of Hh (Thérond et al., 1996; Lum et al., 2003). It has been shown that Hh induces Fu dimerization in a dose-dependent manner, which is mediated by a conformational change and dimerization of Smo (Shi et al., 2011; Zhang et al., 2011). In addition to its association with Cos2, forced dimerization of Fu induces its auto-phosphorylation and the activation of Hh signaling, regulates the ratio of Ci^A (an active form of Ci^F) and Ci^R by interfering with Ci-Sufu and Ci-Cos2-kinase complex formation (Shi et al., 2011; Zhang et al., 2011). Moreover, Thr158 in the activation loop of Fu was identified to be a critical residue phosphorylated upon Hh stimulation (Fukumoto et al.,

2001). In *Drosophila*, Sufu forms a protein complex with Cos2, Fu and Ci, which is reported to regulate Ci subcellular localization and transcriptional activity in the nucleus (Méthot and Basler, 2000; Wang et al., 2000b). Sufu is phosphorylated in response to Hh and this phosphorylation event depends on Fu kinase activity (Lum et al., 2003; Dussillol-Godar et al., 2006), although it needs to be confirmed whether Fu is a direct kinase for Sufu. Recently, Oh et al. found Sufu phosphorylation is irrelevant for pathway regulation, meanwhile Fu and other components regulate Ci transcriptional activity independently of Sufu (Oh et al., 2015), leading to a possible additional layer of regulation.

Smo ciliary accumulation is regulated by phosphorylation. Primary cilium is critical for mammalian Hh signaling (Huangfu et al., 2003; Huangfu and Anderson, 2005; Rohatgi et al., 2007), which can be used as a platform for investigating the mechanism how activators or repressors regulate the signaling pathway. Since Shh or Smo oncogenic mutations promote Smo activation by inducing its ciliary localization (Corbit et al., 2005; Rohatgi et al., 2007), it is interesting whether Smo phosphorylation could regulate its ciliary accumulation. Mammalian Smo does not contain the three PKA/CK1 phosphorylation clusters found in *Drosophila* Smo C-tail, however, Chen et al. found that phosphorylation of mammalian Smo by CK1α and GRK2 at multiple residues in its C-tailpromotes the activation and ciliary localization of Smo (Chen et al., 2011). Differential phosphorylation of vertebrate Smo correlates with the gradient of Hh activity. Similarly, Smo phosphorylation is also induced by oncogenic mutation and the agonist SAG (Zhao et al., 2007), but not by the antagonist cyclopamine that promotes Smo ciliary localization but blocks Smo phosphorylation, conformational change and Hh pathway activation (Zhao et al., 2007; Rohatgi et al., 2009; Wilson et al., 2009). These results suggest that Smo ciliary localization is insufficient for potent Smo activation, whereas Hh induced phosphorylation of Smo is required for the full activation of Smo. Since other components like intraflagellar transport (IFT) proteins (Huangfu et al., 2003; Wang et al., 2009) and β-arrestins (Kovacs et al., 2008) have roles in mediating Smo ciliary localization, the relationship between Smo phosphorylation and its interaction with these components will be an interesting question to investigate.

In mammalian Hh signaling, aPKCι/λ phosphorylates Gli1, promotes Gli1 binding to the target DNA and elevates Gli1 activity, therefore activates Hh signaling (Atwood et al., 2013). *Drosophila* aPKC-Par6 complexalso has a positive role in Hh signaling by phosphorylating Smo, prompting Smo basolateral accumulation and regulating the activity of Ci (Jiang et al., 2014). Considering the similarity between Smo membrane accumulation in *Drosophila* and Smo translocation to cilium in mammalian cells, it is possible that malfunction of aPKC will lead to aberrant Smo ciliary accumulation and activation. Par3/Par6/aPKC polarity complex localizes to cilium and regulates intraflagellar transport and ciliogenesis (Fan et al., 2004; Prulière et al., 2011; He et al., 2012), suggesting an important role for aPKC to associate with microtubule motors in cilium formation and ciliogenesis. Further investigation on the functions of aPKC in the cilium may result in a novel therapeutic approach for the treatment of Hh-mediated tumorigenesis.

Hh may also regulate the dephosphorylation of Hh signaling components that is mediated by their phosphatases. Protein phosphatase 2A (PP2A) was identified as a positive regulator in

Hh signaling (Nybakken et al., 2005; Rorick et al., 2007; Casso et al., 2008), although the relevant substrate remain unidentified. Jia et al. found protein phosphatase 4 (PP4) and PP2A are the phosphatases in regulating dephosphorylation of Smo and Ci, respectively (Jia et al., 2009). The other independent study found that PP2A may also play a role in dephosphorylating Smo (Su et al., 2011). As discussed earlier, multiple Ser/Thr kinases regulate Hh signaling by phosphorylating Smo and Ci, it is not surprisingly that other phosphatases are also involved in dephosphorylating Smo and Ci, as well as other Hh signaling components.

Smo ubiquitination regulated by Hh

As one of the posttranslational modification, protein modification by a small protein called ubiquitin is also involved in Hh signal transduction, which can modulate the stability, activity and sub-cellular localization of the Hh signaling components (Jiang, 2006; Hsia et al., 2015). Unlike the positive role for phosphorylation to regulate Smo, ubiquitination has a negative role in promoting Smo endocytosis, trafficking and degradation in the cell (Li et al., 2012; Xia et al., 2012).

Two independent studies have demonstrated that Smo is poly- and mono-ubiquitinated, leading to the endocytosis and degradation by the lysosome-mediated pathway, which is regulated by Hh stimulation (Li et al., 2012; Xia et al., 2012). Smo ubiquitination also promotes Smo degradation through the proteasome-mediated pathway. Using RNAi screen, Xia et al. identify that ubiquitin-specific protease 8 (USP8) as a deubiquitinase (DUB) required for Hh-induced deubiquitination and cell surface accumulation of Smo. Hh promotes the formation of a Smo-USP8 complex, and USP8 further promotes the accumulation of Smo at the cell surface and prevents localization to the early endosomes by deubiquitinating Smo, leading to increased Hh signaling activity. Importantly, evidence also showed that USP8-mediated elevation of Smo is still inhibited by Ptc, and that phosphorylation and dimerization is required for Smo activation even though Smo can be stabilized by deubiquitination (Xia et al., 2012). Both studies found that Smo ubiquitination is regulated by both Hh and PKA/CK1-mediated Smo phosphorylation. Inactivation of the ubiquitin activating enzyme-Uba1 promotes Smo accumulation on the cell surface and Hh signaling activation, and USP8 decreases Smo ubiquitination to promote its cell surface accumulation in the absence or presence of Hh (Li et al., 2012; Xia et al., 2012).

Since the endosomal sorting complex required for transport (ESCRT) facilitates the ubiquitinated proteins trafficking from endosomes to lysosomes via MVBs (Williams and Urbé, 2007; Wollert and Hurley, 2010), some ESCRT homolog members were found to regulate ubiquitinated Smo in endosomal sorting, like HGF-regulated tyrosine kinase substrate (Hrs) (Li et al., 2012; Fan et al., 2013), Tumor susceptibility gene 101 (Tsg101) (Li et al., 2012) and Vacuolar protein sorting 36 (Vps36) (Yang et al., 2013). Hrs promotes Smo ubiquitination, mediates Smo trafficking in the late endosome (Fan et al., 2013). The N-terminus of Hrs directly interacts with the PKA/CK1 phosphorylation clusters to prevent Smo phosphorylation and activation, indicating an ubiquitin-independent regulation of Smo by Hrs, unlike in many cases that Hrs requires substrate ubiquitination. Knockdown of Tsg101 accumulates Smo in wing discs that is co-localized with Hrs and late endosome

markers-Rab7 and Lamp1, indicate that Tsg101 and Hrs mediate Smo trafficking in the late endosomes (Fan et al., 2013). In mammals, Shh treatment induced a substantial decrease in Smo ubiquitination, suggest a conserved mechanism that regulates Smo (Xia et al., 2012), although more details are still lacking regarding Smo regulation by ubiquitination in mammals.

Although the role of ubiquitination in regulating Smo activity has been documented recently (Hsia et al., 2015), the ubiquitin-protein ligase(s) that directly regulate Smo are still unknown. In an RNAi screen targeting the E3 ligases in the fly genome, Du et al. identified neddylation genes involved in Hh signaling (Du et al., 2011), however, none of the genes in this collection are directly involved in Smo ubiquitination. It is possible that multiple E3 ligases are involved in Smo ubiquitination and degradation by the proteasome- and lysosome-mediated pathways.

Phosphoinositides in Hh signaling

Intraflagellar transport proteins are essential for the assembly and maintenance of cilium required for vertebrate Hh signaling (Rosenbaum and Witman, 2002; Huangfu et al., 2003; Liu et al., 2005). However, the negative role of IFT-A complex subunits in Hh signaling suggests a role of IFT-A complex in additional to regulating ciliogenesis. Tubby-like protein 3 (Tulp3) and IFT-A promote trafficking of the GPCR 161 (GPR161), thus mediate the PKA-dependent basal repression of Hh signaling (Mukhopadhyay et al., 2010; Mukhopadhyay et al., 2013). Endogenous GPR161 is absence in the cilium when the cells are treated with Shh, whereas overexpression of GPR161 resulted in increased cAMP production and therefore PKA activation (Mukhopadhyay et al., 2013). Interestingly, in the *in vitro* protein-lipid overlay assay, GST-TULP3 binds most strongly to PI $(4,5)P_2$ on lipid-dotted strips (Mukhopadhyay et al., 2010), raising the possibility that phosphoinositides (PIs) may have function in the Hh signaling pathway. Although some regulators of Hh signaling are known to be accumulated in the primary cilium, much less is known about the lipids.

PIs are a group of lipids found in various cellular membranes, which have critical functions in regulating numerous biologic processes, including gene expression, protein and membrane trafficking, cell signaling, ion channels, as well as endocytic and exocytic processes (Di Paolo and De Camilli, 2006; Balla, 2013). The inositol ring of phosphatidylinositol, the precursor of PI, can be reversibly phosphorylated at the third, fourth and fifth positions in all combinations, resulting in generating seven different PI species: phosphatidylinositol 3-phosphate ($PI(3)P$), $PI(4)P$, PI $(5)P$, $PI(3,4)P_2$, $PI(3,5)P_2$, $PI(4,5)P_2$ and $PI(3,4,5)P_3$. PIs are enriched on specific subcellular membranes, as $PI(4,5)P_2$ and $PI(3,4,5)P_3$ are concentrated at the plasma membrane, $PI(3,4) P_2$ is mostly found at the plasma membrane and in the early endocytic pathway, $PI(4)P$ is enriched at the Golgi complex, but also present at the plasma membrane, $PI(3)P$ is concentrated in early endosomes, while $PI(3,5)P_2$ is formed at the multivesicular body and $PI(3,5)P_2$ concentrated on late endosomal pathway (Di Paolo and De Camilli, 2006; Balla, 2013).

Inositol polyphosphate 5-phosphatase E (INPP5E) is known to remove the 5-phosphate from $PI(4,5)P_2$, $PI(3,4,5)P_3$, and $PI(3,5)P_2$. INPP5E has a proline-rich sequence and a COOH-terminal CAAX box in addition to the 5-phosphatase domain, which locates mostly in the Golgi and partially in the plasma membrane. Evidence showed that mutations in the phosphatase domain of INPP5E promoted premature destabilization of cilia in response to stimulation, hence are responsible for Joubert syndrome (Bielas et al., 2009). INPP5E mutation also affect its ciliary localization and cilium stability in a family with MORM syndrome (Jacoby et al., 2009). The physical targeting of INPP5E to the cilium is dependent on a protein-protein interaction network included the ADP-ribosylation factor-like 13B (ARL13B), phosphodiesterase 6D (PDE6D), and the centrosomal protein 164 (CEP164) (Humbert et al., 2012). All of them have been linked to ciliopathies, kind of disease associated with ciliary dysfunction (Hildebrandt et al., 2011). These findings indicate a role for PIs to regulate the function of cilium. Recently, two studies show that INPP5E restricts the ciliary PIs trafficking and accumulation of negative regulators of Hh signaling (Chávez et al., 2015; Garcia-Gonzalo et al., 2015). Chávez et al. identified that the main PI in neural stem cell primary cilium membrane is $PI(4)P$, and that $PI(4,5)P_2$ is limited to the ciliary base (Chávez et al., 2015). They also showed that the INPP5E regulates PI distribution, as in INPP5E knockout neuronal stem cells primary cilium $PI(4,5)P_2$ is detected and $PI(4)P$ is absence, and that this regulation depends on its phosphatase activity. INPP5E affects recruitment of the $PI(4,5)P_2$-interacting protein Tulp3, as well as Hh signaling repressor IFT-A components and Gpr161, thus regulates Hh signaling (Chávez et al., 2015). The same conclusion was made by Garcia-Gonzalo et al. in mouse embryonic fibroblasts (MEFs) with a slightly different approach (Garcia-Gonzalo et al., 2015), meanwhile they found INPP5E promotes Hh signal at a step subsequent to Smo ciliary localization and prior to Gli3 accumulation at the ciliary tip.

Ptc is not physically associated with Smo because they do not appear to be enriched at similar subcellular locations *in vivo* (Jia and Jiang, 2006; Jiang and Hui, 2008). Ptc acts indirectly to inhibit Smo activity, instead functions as a permease to facilitate transmembrane movement of a small molecule(s) that acts as Smo agonist or antagonist (Taipale et al., 2002). Evidence indicates that, in *Drosophila*, downregulation of the Stt4 kinase or upregulation of the Sac1 phosphatase keep the $PI(4)P$ at low level in cells, which results in a phenotype similar to that of loss of Ptc (Yavari et al., 2010). Taken together of the recent published studies (Yavari et al., 2010; Chávez et al., 2015; Garcia-Gonzalo et al., 2015), it is still unknown whether phospholipids directly regulate the key Hh signaling regulators, Ptc and Smo.

In a most recent study, Jiang et al. found that Hh promotes the production of $PI(4)P$ in both wing discs and cultured cells (Jiang et al., 2016) (Fig. 2). In both *Drosophila* S2 cells and mammalian NIH3T3 cells, $PI(4)P$ was detected by the sensitive direct mass-spec assay and the levels of $PI(4)P$ are elevated by Hh stimulation. Also in cultured cells, $PI(4)P$ stimulates the phosphorylation and dimerization of Smo through direct interaction with an arginine motif in Smo, which is conserved between *Drosophila* Smo and mammalian Smo, as such motifs have been mapped in Smo species (Zhao et al., 2007). Mutation in the R4 arginine motif abolishes PI (4)P-Smo direct interaction, indicated that Smo conformation change due

to the binding of PI(4)P to the specific arginine motif is critical. The position for PI(4)P binding is also critical to trigger Smo activation, because fusion of a pleckstrin homology (PH) domain to either the 3rd intracellular loop or the C terminus of Smo attracts PI(4)P to different locations inside Smo, thus blocking Smo activation by PI(4)P. Surprisingly phospho-mimetic Smo mutations (Smo^{SD123} and $mSmo^{SD}$) are still regulated by PI(4)P, suggesting that PI (4)P either promotes phosphorylation at other residues or has additional role(s) in activating Smo (Jiang et al., 2016).

Gprk2 is positively involved in Hh signaling by directly phosphorylating Smo, and by forming a dimer to promote Smo dimerization and activation in a kinase activity-independent manner (Chen et al., 2010). But how Gprk2 promotes Smo dimerization and activation is not clear. Jiang et al. demonstrate that the PH domain of Gprk2 is responsible to enrich PI(4)P, which promotes Smo phosphorylation and dimerization. The findings that lipids bind to the PH domain promotes protein dimerization (Swanson et al., 2008; Zwolak et al., 2013), leading to the possibility for PI(4)P to promote Gprk2 dimerization, eventually promoting Smo phosphorylation and dimerization (Chen et al., 2010).

Hh likely promotes the production of PI(4)P by regulating the activity of PI(4)P kinase Stt4 and phosphatase Sac1 (Jiang et al., 2016), which may account for the mechanism by which Ptc acts catalytically to inhibit Smo. In this recent study, an additional layer of regulation has been identified, in which Hh may regulate the release of PI(4)P from Ptc in order for PI(4)P to activate Smo (Jiang et al., accepted). The sterol-sensing domain (SSD)of Ptc strongly interacts with PI(4)P, raising the possibility that Ptc may control the pool of phospholipids in regulating the accessibility of Smo to PI(4)P. Indeed, Hh treatment increases Smo-PI(4)P interaction however decreases Ptc-PI(4)P interaction, therefore binding of Hh to Ptc may cause the conformation change, leading to the release of PI(4)P from Ptc. The regulation of Smo by PI(4)P is likely conserved between *Drosophila* and vertebrate. PI(4)P promotes vertebrate Smo phosphorylation, activation and localization in the cilium. Meanwhile, PI(4)P prevents the ciliary accumulation of Ptc1 and Ptc2. This interesting discovery should facilitate further studies to better understand the direct role of phospholipids in Hh signaling pathway, ciliary function and how related regulators are controlled in this process.

Concluding remarks

Many studies regarding Smo regulation demonstrate that the mechanisms are highly conserved among species, although *Drosophila* and mammalian Smo have big difference in sequence homology in the carboxyl-terminal tail (Arensdorf et al., 2015). Smo also has other functional domains as GPCR, including N-terminal cysteine rich domain (CRD), three extracellular, three intracellular loops, and seven transmembrane domains. Recent structural analysis of the Smo N-terminal have suggested that Smo CRD may serve as binding site for exogenous ligand, such as cyclopamine and oxysterols (Myers et al., 2013; Nachtergaele et al., 2013; Rana et al., 2013). It is unknown whether the N-terminal of Smo also has posttranslational modifications. A recent study found that loss of N-glycosylation disrupted Smo trafficking and attenuated its signaling capability (Marada et al., 2015), however, more evidence may be needed for a better understanding.

Studies have shown that USP8 also interacts with and deubiquitinate Hrs (Zhang et al., 2014; Pradhan-Sundd and Verheyen, 2015), demonstrating multiple roles of USP8 in both cargo de-ubiquitination and ESCRT-0 stability during development, which is helpful to address the mechanisms of Hh signaling. Besides, it is interesting to test whether USP8 or other DUBs are involved in reversing the ubiquitination of other Hh signaling components. Balmer et al. performed an RNAi screen in *Drosophila* non-ciliated cells to test whether cilium-associated proteins have cilium-independent functions, and the results show there is no effect on Hh signaling (Balmer et al., 2015), indicating that cilium-associated regulators need to reach the primary cilium to modulate Hh signaling. Whether the DUBs required to be localized in the cilium in order to modulate Hh signaling is unknown.

Studies on the function of endogenous sterol derivatives in Hh signaling also suggest that lipids synthesis and trafficking may regulate not only Hh secretion and spreading, but the activation and trafficking of Hh signaling components (Callejo et al., 2008; Eaton, 2008; Khaliullina et al., 2009). Because recent work has shown that PI(4)P can modulate Hh signaling through regulating Smo, and because PIs have critical roles in regulating cell physiology, signaling, metabolism and membrane trafficking, it is possible for other phospholipids to positively or negatively regulate Hh signaling activity. PI(4)P acts likely in between Ptc and Smo to promote Hh signaling. Further investigation of the effects for phospholipids in the regulating Smo and other Hh components may yield important insights into the intracellular trafficking and activation of Smo that is the key for Hh signaling.

Acknowledgments

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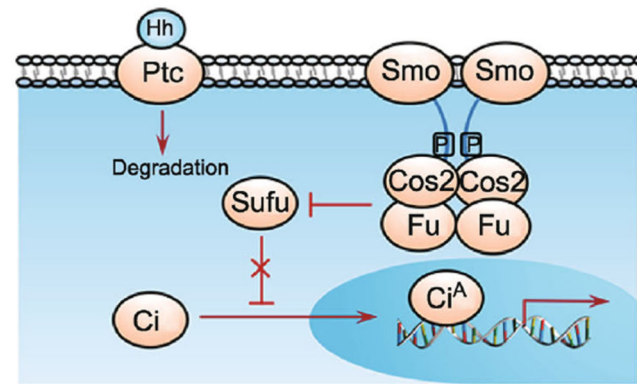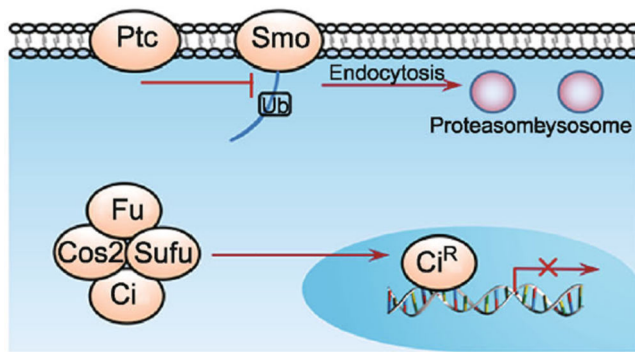
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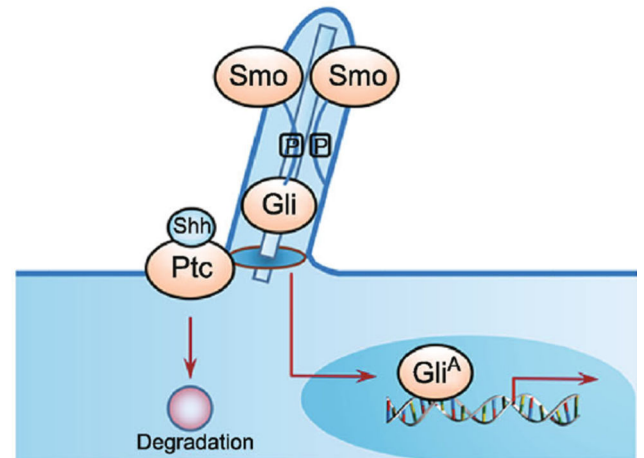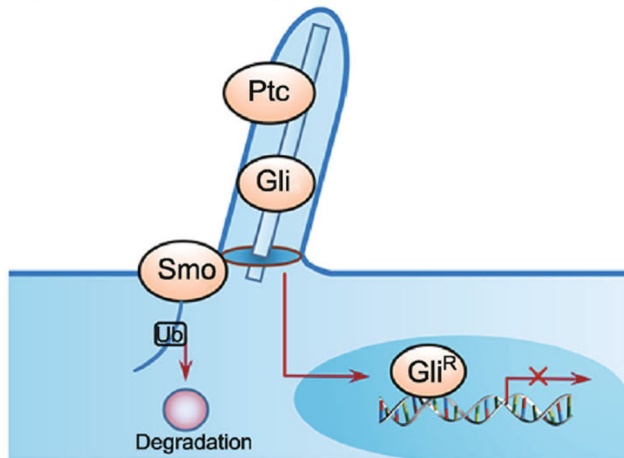
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Figure 1.

The regulation of Smo phosphorylation and ubiquitination in *Drosophila* and mammalian Hh signaling pathways. (A) Schematic drawings of *Drosophila* Hh signaling. In the absence of Hh (left panel), Ptc inhibits Smo. Smo is ubiquitinated at multiple residues among its C-tail, resulting in endocytosis and degradation by both lysosome- and proteasome-dependent pathways. Full-length Ci (Ci^F) undergoes proteolytic processing to generate a truncated repressor form (Ci^R), which blocks the expression of Hh target genes. In the presence of Hh (right panel), binding of Hh to Ptc releases its inhibition on Smo and triggers phosphorylation of Smo by multiple kinases (PKA, CK1, CK2, Gprk2 and aPKC), leading to Smo cell surface accumulation, dimerization and activation, thus the conversion of Ci^F into the activator form Ci^A. (B) Schematic drawings of mammalian Hh signaling in the cilium. In the absence of Shh (left panel), Smo is excluded from the primary cilium, where Ptc resides to inhibit Hh signaling. In the presence of Shh (right panel), binding of Shh to Ptc leading its departure from primary cilium, where Smo is accumulated, phosphorylated, dimerized and activated. The active forms of Gli (Gli^A) induce Hh target gene expression.
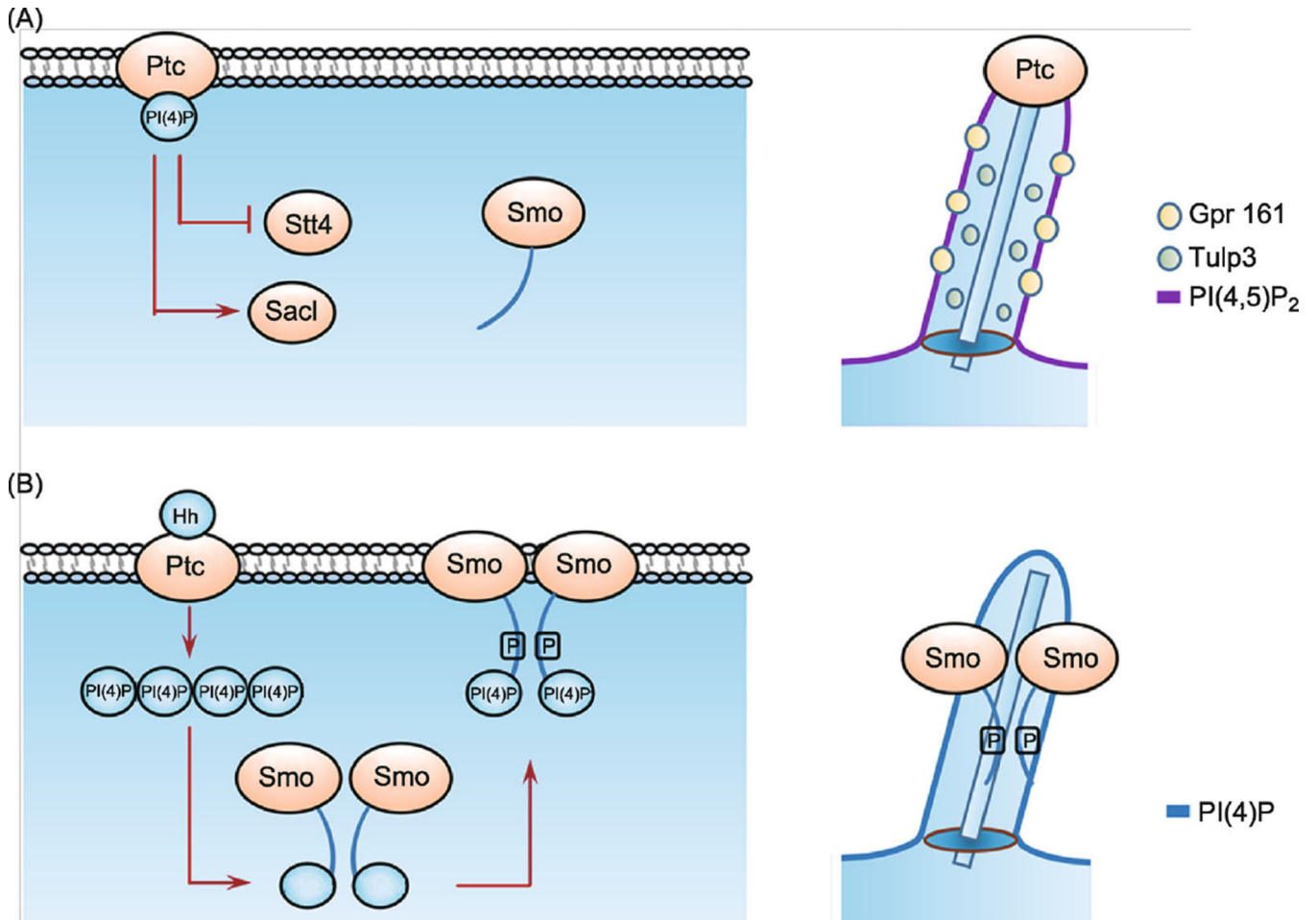
Figure 2.
PI(4)P regulates the phosphorylation and ciliary accumulation of Smo. Models are based on studies from recent publications. (A) Smo regulation in the absence of Hh. In *Drosophila* (left panel), Stt4 has low whereas Sac1 has high activity, resulting in low levels of PI(4)P that interacts with Ptc. In vertebrate cilium (right panel), Ptc1/2 and Grp161 localize in the cilium to inhibit Hh signaling. PI(4,5)P$_2$ accumulates in the cilium but PI(4)P does not. (B) Smo regulation in the presence of Hh. In *Drosophila* (left panel), Hh promotes the production of PI(4)P by activating Stt4 and inactivating Sac1. PI(4)P is also released from Ptc upon Hh stimulation, allowing PI(4)P to bind and activate Smo through promoting Smo phosphorylation and dimerization. In the vertebrate cilium (right panel), PI(4)P levels are elevated by Shh, which promote Smo phosphorylation and ciliary accumulation. PI(4)P elevated by Shh inhibits the ciliary localization of Ptc1/2.