Development 137, 2079-2094 (2010) doi:10.1242/dev.045021

Mechanism and evolution of cytosolic Hedgehog signal transduction

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Summary

Hedgehog (Hh) signaling is required for embryonic patterning and postnatal physiology in invertebrates and vertebrates. With the revelation that the primary cilium is crucial for mammalian Hh signaling, the prevailing view that Hh signal transduction mechanisms are conserved across species has been challenged. However, more recent progress on elucidating the function of core Hh pathway cytosolic regulators in *Drosophila*, zebrafish and mice has confirmed that the essential logic of Hh transduction is similar between species. Here, we review Hh signaling events at the membrane and in the cytosol, and focus on parallel and divergent functions of cytosolic Hh regulators in *Drosophila* and mammals.

Key words: Hedgehog, Evolution, Mechanism, Signaling

Introduction

In embryonic development and postnatal life, a limited number of signal transduction pathways are repeatedly used both to provide instruction to naïve fields of cells and to control differentiation and regeneration. The Hedgehog (Hh) signal transduction pathway is an evolutionarily conserved signaling cascade that is essential for the proper patterning and development of tissues in metazoan organisms (Hooper and Scott, 2005; Huangfu and Anderson, 2006; Jiang and Hui, 2008; Lum and Beachy, 2004). The misregulation or mutation of essential core components of the Hh pathway often result in congenital birth defects, such as polydactyly and holoprosencephaly (McMahon et al., 2003). In adults, the inappropriate activation of Hh signaling leads to cancer, the most common type being basal cell carcinoma (McMahon et al., 2003; Scales and de Sauvage, 2009).

Hh ligands function as morphogens that signal both at short range and over many cell diameters. The interpretation of such Hh ligand concentration gradients requires sophisticated cytosolic and transcriptional transducers (Table 1) that can produce a proportionate response. The mutation of these effectors in the mouse neural tube, for example, is sufficient to drastically perturb graded specification of interneurons and motoneurons (Bai et al., 2004; Wijgerde et al., 2002), and typically results in production of a more limited array of cell types (Briscoe, 2009).

Unlike other major signaling pathways (such as Notch, Fgf and Wnt), the core signaling genes in the Hh pathway have not undergone extensive gene duplication in mammalian lineages (Pires-daSilva and Sommer, 2003). Despite this, the prevailing hypothesis in the field was, until recently, that the molecular mechanism of Hh transduction in responding cells differs significantly between *Drosophila* and mammals (Huangfu and Anderson, 2006; Varjosalo et al., 2006). Here, we review current insights into the molecular mechanisms of Hh signaling that inform us about the conservation and evolution of cytoplasmic signaling events in *Drosophila* and mouse. As we discuss, the new discoveries that we review here, particularly those concerning cytosolic Hh signal transduction and modulation of the Ci/Gli transcription factors, contradict the recent view that core events in Hh transduction diverge in different species.

Hedgehog signal reception and transduction

The basic scheme of Hh morphogen production, movement and transduction in receiving cells is conserved among several model organisms (Eaton, 2008; Farzan et al., 2008; Guerrero and Chiang, 2007) (Fig. 1). Below, we provide an overview of the key steps in the binding of Hh ligand to its receptor and the downstream cytosolic events. This section will highlight the mechanisms in these processes that are conserved between *Drosophila* and mice.

In responding cells, Hh binds to its core receptor Patched (Ptc/Ptch/Ptch1), a twelve-pass SSD transmembrane protein (Marigo et al., 1996a; Stone et al., 1996). Ihog/Cdo proteins function as co-receptors with Ptc and are important for Hh signal transduction (Tenzen et al., 2006; Yao et al., 2006; Zhang, W. et al., 2006; Zheng et al., 2010). In the absence of Hh ligand, Ptch represses the activity of the seven-pass transmembrane protein Smoothened (Smo), a member of the G-protein-coupled receptor (GPCR) superfamily (Fig. 1). The mechanism by which Ptch represses Smo is currently unknown. Early reports that Ptch inhibited Smo by directly binding to it were shown to be overexpression artifacts, and Smo inhibition is achieved by sub-stoichiometric amounts of Ptch (Stone et al., 1996; Taipale et al., 2002). Sequence analysis places Ptch in the resistance-nodulation cell division (RND) superfamily of permeases and transporters; consistent with this, both truncated and full-length forms of *Drosophila* Ptc can trimerize (Lu et al., 2006). Ptch is thought to inhibit Smo by mediating the transport of a lipid-derived molecule (Taipale et al., 2002), either by increasing local concentrations of an inhibitor or decreasing levels of an activator (Eaton, 2008). The binding of Hh to Ptch could disrupt the transport of this small molecule, perhaps by dispersing or inactivating the Ptch oligomer.

Once Smo inhibition is released, it becomes activated by means that are poorly characterized biochemically. In *Drosophila*, conformational changes in Smo are communicated to the cytosol through an Hh signaling complex that comprises the Costal2 (Cos2), Fused (Fu), and Suppressor of fused [Su(fu)] proteins (Table 1). The result of Smo activation is modulation of the repressor and activator forms of the Ci/Gli zinc-finger transcription factors (Ci in *Drosophila*; Gli1-3 in mammals). In the 'off' (Smo-inhibited) state of the pathway, Ci/Gli2/Gli3 are phosphorylated by protein kinase A (PKA), casein kinase I (CKI) and glycogen synthase kinase 3 (GSK3), targeting the proteins for proteasome-dependent processing (Bhatia et al., 2006; Jia et al., 2002; Jia et al.,

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Table 1. Core components of the *Drosophila*, zebrafish and mouse Hedgehog (Hh) pathway

Drosophila gene	Zebrafish homolog	Mouse homolog	Function	Conserved?	Key references
Hedgehog (*Hh*)	*Shh*, *Twhh*, *Ehh*, *Ihh*, *Dhh*	*Shh*, *Ihh*, *Dhh*	Signaling ligand	Yes	Chiang et al., 1996; Nusslein-Volhard and Wieschaus, 1980; Bitgood et al., 1996; Porter et al., 1996a; St-Jacques et al., 1999; Tabata and Kornberg, 1994; http://www.zfin.org
Skinny hedgehog (*Ski*)	*Hhat*	*Hhat* (*Skn*)	Palmitoylates Hh ligands	Yes	Chamoun et al., 2001; Chen, M. H. et al., 2004; Pepinsky et al., 1998
Dispatched (*Disp*)	*Disp1*, *Disp2*	*Disp1*, *Disp2*	Hh ligand release	Yes	Burke et al., 1999; Caspary et al., 2002; Kawakami et al., 2002; Ma et al., 2002
Patched (*Ptc*)	*Ptc1*, *Ptc2*	*Ptch1*, *Ptch2*	Inhibits Smo	Yes	Goodrich et al., 1997; Nusslein-Volhard and Wieschaus, 1980; Johnson et al., 1996; Stone et al., 1996
Interference hedgehog (*Ihog*), *Brother of interference hedgehog* (*Boi*)	*Cdo*, *Boc*	*Cdo*, *Boc*	Co-receptors with Ptc	Yes	Tenzen et al., 2006; Yao et al., 2006; Zhang, W. et al., 2006; Zheng et al., 2010
Smoothened (*Smo*)	*Smo*	*Smo*	Positive membrane transducer	Yes	Alcedo et al., 1996; van den Heuvel and Ingham, 1996; Zhang et al., 2001
Costal2 (*Cos2*)	*Kif7*	*Kif7*	Scaffold for Ci/Gli processing, positive and negative roles	Yes	Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009; Robbins et al., 1997; Sisson et al., 1997; Tay et al., 2005
Fused (*Fu*)	*Fu*	*Fu* (*Stk36*)	Required for Cos2 and Sufu phosphorylation, positive transducer	No	Chen et al., 2005; Merchant et al., 2005; Nusslein-Volhard and Wieschaus, 1980; Préat et al., 1990; Thérond et al., 1996; Wolff et al., 2003
Suppressor of fused [*Su(fu)*]	*Sufu*	*Sufu*	Protects Ci/Gli proteins from HIB/Spop-induced degradation, negative regulator	Yes	Chen et al., 2009; Cooper et al., 2005; Koudijs et al., 2005; Préat, 1992; Svärd et al., 2006; Wolff et al., 2003
Cubitus interruptus (*Ci*)	*Gli1*, *Gli2a*, *Gli2b*, *Gli3*	*Gli1*, *Gli2*, *Gli3*	Transcriptional activator and repressor	Yes, but partitioned*	Hui and Joyner, 1993; Hui et al., 1994; Sasaki et al., 1999

Twhh, Tiggy-winkle hedgehog; Ehh, Echidna hedgehog; Ihh, Indian hedgehog; Dhh, Desert hedgehog; Hhat, Hedgehog acyltransferase; Cdon, CAM-related/down-regulated by oncogenes; Boc, Brother of Cdo; Kif7, Kinesin family member 7; Stk36, Serine-threonine kinase 36; Spop, Hedgehog-induced MATH and BTB domain-containing protein or Speckle-type POZ protein.
*Gli1 – activator, not proteolytically processed; Gli2 – activator, undergoes inefficient processing to repressor; Gli3 – repressor and weak activator, efficiently processed to repressor.

2005; Pan et al., 2006; Price and Kalderon, 2002; Wang and Li, 2006). This processing event eliminates the C-terminal transactivation domains from full-length Ci/Gli2/Gli3, thus forming a transcriptional repressor that comprises the DNA-binding zinc-finger domains of Ci/Gli2/Gli3 and a poorly characterized N-terminal repression domain (Aza-Blanc et al., 1997). Smo activation inhibits Ci/Gli2/Gli3 proteolysis and might promote the formation of biochemically undefined Ci/Gli activators from the full-length proteins (Methot and Basler, 2001; Smelkinson et al., 2007). The relative ratio of Ci/Gli full-length and repressor forms is considered to be crucial for interpreting the extracellular Hh gradient and for determining concentration-dependent cell fates.

Communication from Smo to Ci/Gli is a crucial step in Hh signal transduction that is tightly regulated. In both *Drosophila* and mammals, two general principles have emerged from studying Ptch and Smo trafficking. First, the opposite subcellular localization of Ptch and Smo at the cell surface or in intracellular membranes is associated with the off and on (Hh-bound) states of the pathway (Denef et al., 2000; Rohatgi et al., 2007). Second, Smo conformational changes are required for Hh pathway activation (Zhao et al., 2007) and are coupled to downstream factors via scaffolds that relay the signal to Ci/Gli (Aikin et al., 2008). Below, we discuss species-specific differences and common mechanisms of action that have been identified between *Drosophila* and mammalian Hh signaling.

Drosophila Smo trafficking and conformational change

Studies of Ptc and Smo localization in the *Drosophila* wing imaginal disc and salivary gland have revealed that a complex interplay exists between their trafficking and stability of these two proteins. In the absence of Hh, Ptc is found both on the plasma membrane and in perinuclear and cytosolic intracellular compartments (Denef et al., 2000; Zhu et al., 2003). Ptc inhibits Smo by both promoting its turnover and preventing its accumulation at the cell surface (Denef et al., 2000) (Fig. 2A). Changes in Smo localization are associated with Hh pathway activation. For example, increasing the level of cell-surface Smo correlates well with activation of Hh signaling (Nakano et al., 2004; Zhao et al., 2007; Zhu et al., 2003). Conversely, the forced retention of Smo in the endoplasmic reticulum (ER) prevents ectopic activation of the pathway. In addition, when Hh binds to

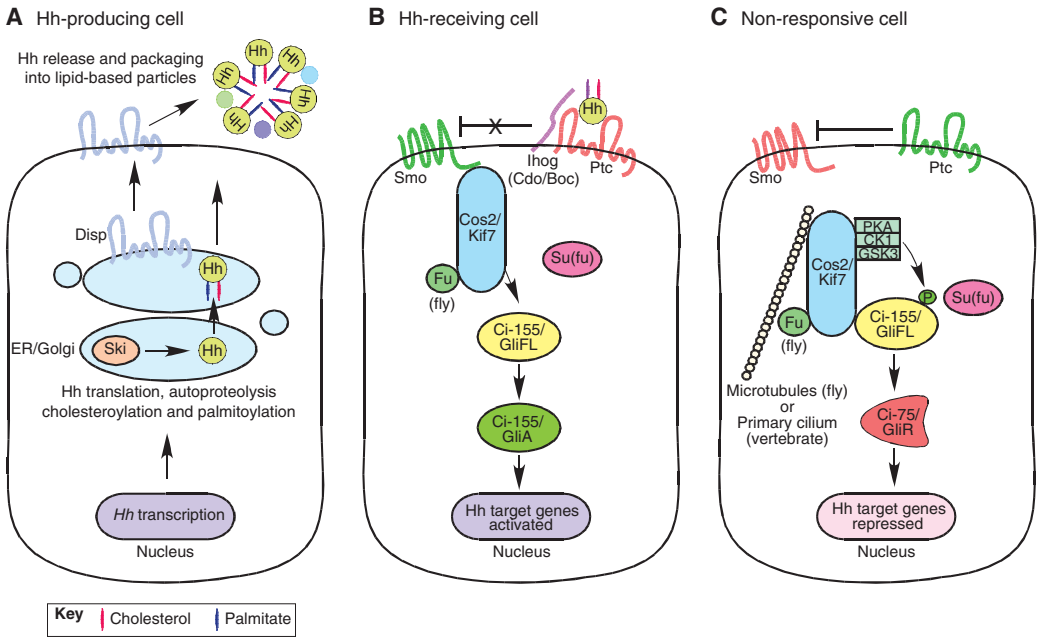

Fig. 1. Schematic of Hh production and reception. (**A**) In mice and *Drosophila*, Hh is synthesized as an ~45 kilodalton (kDa) precursor, which is targeted to the endoplasmic reticulum (ER) and Golgi (Lee et al., 1994). Hh then undergoes autoproteolytic cleavage and cholesterol (pink) addition catalyzed by its C-terminal intein domain (Bumcrot et al., 1995; Porter et al., 1996a; Porter et al., 1996b). Palmitate (blue) is attached to its N-terminus by the acyltransferase Skinny hedgehog (Ski/Skn/Hhat) (Chen, M. H. et al., 2004; Steinhauer and Treisman, 2009). Palmitate is important for high-level Hh signaling, whereas cholesterol functions in Hh oligomerization or its packaging into signaling complexes or particles (Chen, M. H. et al., 2004; Panakova et al., 2005; Zeng et al., 2001) and restricts its movement in morphogenetic fields (Li et al., 2006; Porter et al., 1995). During its trafficking and release, Hh is packaged into lipid-associated particles, and its release from Hh-producing cells is facilitated by the sterol-sensing domain (SSD) protein Dispatched (Disp/Disp1) (Burke et al., 1999; Caspary et al., 2002; Etheridge et al., 2010; Kawakami et al., 2002; Ma et al., 2002). (**B**) In responding cells, Hh binding to the Ptc/Ihog/Boi (Ptch1/Cdo/Boc) co-receptor alleviates Ptc inhibition of Smo, which results in release of the transcription factor Ci-155 (or Gli1-3) from a cytosolic complex comprising Cos2, Fu and Su(fu) (in vertebrates, this complex is less well characterized). Activated Ci/Gli translocates to the nucleus to activate Hh target genes. (**C**) In cells not receiving Hh ligand, Ptc inhibits Smo activity. The cytosolic complex, comprising Cos2, Fu and the kinases PKA, CK1 and GSK3, promotes the proteolytic processing of Ci-155 by phosphorylating it, converting it into a transcriptional repressor (Ci-75, red). Ci-75 represses Hh target genes in the nucleus. In some instances, cells might sense distant Hh ligand through long, actin-based cellular extensions known as cytonemes (Ramirez-Weber and Kornberg, 1999). A, activator; Ci, Cubitus interruptus; CKI, casein kinase I; Cos2, Costal2; Disp, Dispatched; ER, endoplasmic reticulum; FL, full-length; Fu, Fused; GSK3, glycogen synthase kinase 3; Hh, Hedgehog; Ihog, Interference hedgehog; Kif7, Kinesin family member 7; P, phosphate group; PKA, protein kinase A; Ptc, Patched; R, repressor; Ski, Skinny hedgehog; Smo, Smoothened; Su(fu), Suppressor of fused.

Ptc, an Hh-Ptc complex forms that moves from the cell surface into intracellular vesicles where no significant colocalization with Smo is observed (Denef et al., 2000; Incardona et al., 2002) (Fig. 2B). Thus, a crucial role for Ptc is to control Smo trafficking (Martin et al., 2001).

The exposure of cells to exogenous Hh or to a reduction in Ptc activity promotes the stabilization of Smo and the hyperphosphorylation of its intracellular C-terminal tail (Zhang et al., 2004). This region of Smo contains several consensus PKA phosphorylation sites, as well as CKI sites that require prior PKA phosphorylation; up to a total of 26 serine/threonine (Ser/Thr) residues in this region may be modified (Jia et al., 2004; Zhang et al., 2004). Mutagenesis studies in *Drosophila* that mimic the gain and loss of this phosphorylation have shown that graded activity of Smo correlates with the extent of its C-terminal phosphorylation (Jia et al., 2004; Zhang et al., 2004). Furthermore, phosphorylated Smo accumulates at the cell surface, consistent with its ability to activate the Hh pathway (Fig. 2). Phosphorylation of Smo has thus been proposed to inhibit its endocytosis or to promote its rapid recycling between endosomal vesicles and the cell surface.

How does Smo phosphorylation lead to its activation? Fluorescence resonance energy transfer (FRET) studies of Smo conformation have illuminated the role that clusters of positively charged arginine (Arg) and lysine (Lys) residues might play in Smo activation (Zhao et al., 2007). Adjacent to these residues are PKA/CKI phosphorylation sites that create a negative electrostatic charge when phosphorylated, which neutralizes the inherent positive charge of the Arg clusters (Fig. 3A). Smo is a constitutive dimer and, within the homodimer, the Arg clusters interact with acidic residues in the Smo C-terminal tail to keep the molecule in a ‘closed’ conformation (Fig. 3A). Phosphorylation of the PKA/CKI clusters disrupts these intramolecular Smo interactions and promotes formation of an ‘open’ conformation (Fig. 3A). In the open conformation, intermolecular interactions form between the Smo C-termini in the constitutive dimer, resulting in pathway activation, potentially by coupling to downstream components of the *Drosophila* Cos2 complex. The graded nature of Smo C-tail phosphorylation might allow variable amounts of extracellular Hh ligand to be interpreted to promote a proportionate Ci response. The general principles of Smo conformational change, trafficking and the role

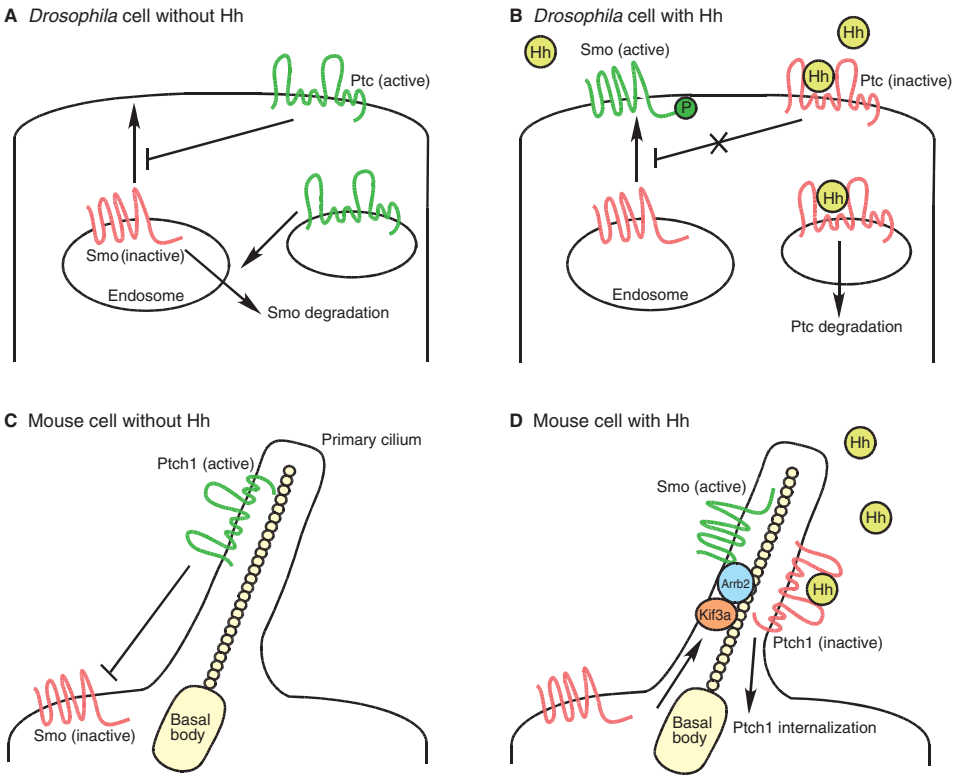

Fig. 2. Smo and Ptch trafficking without and with the Hh ligand. Smo and Ptch have opposite localizations. Red denotes an inactive protein and green an active protein. (**A**) In *Drosophila*, Smo is found in endosomes and vesicles, and Ptc prevents Smo trafficking to the cell surface, while also promoting its degradation. (**B**) When Hh ligand is sensed and binds to Ptc, the Ptc-Hh complex is internalized and degraded. Smo is phosphorylated (P) on its C-terminus and moves to the cell surface to activate downstream effectors. (**C**) In mice, Hh signaling utilizes the primary cilium. In the absence of Hh ligand, Ptch1 is found on the primary cilium and normally prevents Smo from translocating to the cilium to activate signaling. (**D**) After binding of Hh ligand to Ptch1, the complex moves off the cilium, allowing Smo to move along the axoneme in a Kif3a- and β-arrestin2-dependent manner. Arrb2, β-arrestin2; Hh, Hedgehog; Ptc/Ptch1, Patched; Smo, Smoothened.

of the Arg clusters are conserved in mammals (Zhao et al., 2007), although the precise subcellular locations where these events occur might have changed during evolution (Fig. 3B).

Mammalian Smo trafficking, conformation and the primary cilium

Hh signal transduction in mammals utilizes the primary cilium, an evolutionarily conserved microtubule-based organelle analogous to the flagella found in single-celled eukaryotes, such as *Chlamydomonas reinhardtii* (Berbari et al., 2009; Eggenschwiler and Anderson, 2007; Gerdes et al., 2009). The assembly and disassembly of the cilium is mediated by intraflagellar transport (IFT) proteins and their associated kinesin II (Kif3 family) and dynein motors (Rosenbaum and Witman, 2002). Mice deficient in genes essential for cilium assembly and maintenance, such as *Kif3a* and *Ift88*, display a loss of both Gli repressor and activator function in vivo, implicating the primary cilium in the reception and interpretation of Hh signals (Huangfu and Anderson, 2005; Huangfu et al., 2003; Liu et al., 2005; May et al., 2005). Analyses of endogenous and overexpressed Smo, Ptch1, Gli1, Gli2, Gli3 and Suppressor of fused (Sufu), all core components of vertebrate Hh signaling (Table 1), have indicated that these proteins localize to the primary cilium (Chen et al., 2009; Corbit et al., 2005; Haycraft et al., 2005; Rohatgi et al., 2007). The dynamic trafficking of endogenous Ptch1, Smo, Gli2 and Gli3 has been observed at various time points after Hh stimulation (Chen et al., 2009; Corbit et al., 2005; Rohatgi et al., 2009; Rohatgi et al., 2007; Wang et al., 2009; Wilson et al., 2009a). Cultured cells that lack cilia, such as *Kif3a*-null mouse embryonic fibroblasts (MEFs), are refractory to stimulation by exogenous Hh ligands, and the overexpression of constitutively active forms of Smo or the treatment of these cells with Smo agonists fails to activate the pathway in the absence of the cilium (Chen et al., 2009; Ocbina et al., 2009). By contrast, primary cilia do not seem to be involved in *Drosophila* Hh signaling. In *Drosophila*, only a few cell types are ciliated (sensory neurons and spermatozoa), and IFT mutants do not exhibit altered Hh signaling (Han et al., 2003; Sarpal et al., 2003). Further studies of the mechanism of Hh signal transduction in additional metazoan model organisms are needed to address whether the primary cilium was involved in ancestral Hh signaling or whether it is a later evolutionary acquisition in vertebrate or mammalian lineages (Glazer et al., 2010; Rink et al., 2009).

Despite the apparent divergence in the subcellular location of Hh transduction between *Drosophila* and mammals, the general principles of Smo regulation by Ptch and of Smo conformational change in response to Hh are similar and/or comparable in *Drosophila* and mice (Zhao et al., 2007). In the absence of Hh ligand, mammalian Ptch1 is found on the primary cilium and might serve as a concentrated local sensor for extracellular ligand concentration (Rohatgi et al., 2007) (Fig. 2C). The binding of Hh to Ptch1 causes the removal of Ptch1 from the primary cilium (Rohatgi et al., 2007) (Fig. 2D). Concomitantly, Smo translocates to the cilium in a Kif3a- and β-arrestin (a GPCR regulator)-dependent manner (Corbit et al., 2005; Kovacs et al., 2008), either from lateral regions of the plasma membrane or by being directly trafficked from the Golgi (Milenkovic et al., 2009; Wang et al., 2009). The opposing translocation of Ptch1 and Smo can be decoupled by modulating Smo conformation. The treatment of MEFs or NIH 3T3 cells with the Smo antagonist cyclopamine, the Smo agonist SAG or the pathway agonist 20-α-hydroxysterol results in Smo trafficking to the cilium, without the corresponding removal of Ptch1 from the cilium (Rohatgi et al., 2009; Rohatgi et al., 2007; Wang et al., 2009; Wilson et al., 2009a). Thus, the movement of Smo to the primary cilium appears to be necessary, but not sufficient, for signal transduction (Fig. 3B). Furthermore, the presence of Ptch1 on the cilium is less a crucial determining factor for pathway status than is the conformation of Smo, as both inactive and

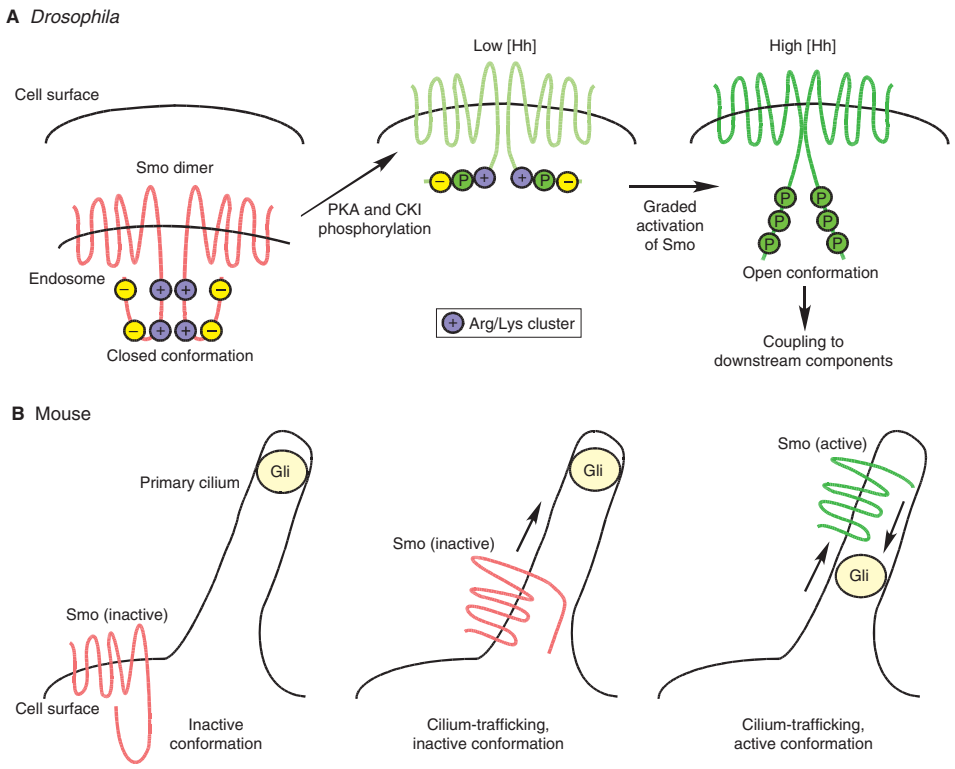

Fig. 3. Smo conformational changes, trafficking and pathway activation. Smo exists as a constitutive dimer and conformational changes accompany its trafficking and activation. (**A**) (Left) In *Drosophila*, inactive Smo (red) cycles within the cell and the protein is in a closed conformation. Positive charges from the Arg clusters (purple circles) in the Smo C-tail are neutralized by distal acidic residues (yellow circles). (Middle) As increasing concentrations of Hh ligand bind to Ptc, the Smo C-tail is phosphorylated by PKA and CKI. This leads to local neutralization of the Arg clusters through phosphorylated Ser and Thr residues (green circles) and the movement of Smo to the cell surface. (Right) High Hh concentrations cause full phosphorylation and increased proximity of Smo C-tails, which may lead to an open conformation that might facilitate the coupling of Smo to the Hh signaling complex. (**B**) (Left) In mice, Smo is in an inactive conformation (red) in the absence of Hh. (Middle) Smo adopts a conformation that permits trafficking to the primary cilium, but does not activate the pathway. This is recapitulated by binding of the veratrum alkaloids cyclopamine and jervine to Smo. (Right) Removal of Ptch1 or activation of Smo by agonist binding (e.g. to purmorphamine) facilitates cilium trafficking and Smo activation (green), thus permitting Smo to communicate with the Gli proteins. Mouse Smo also dimerizes, but the relationship of Smo dimers to intracellular trafficking is currently unclear in this system. Arg, arginine; CKI, casein kinase I; Hh, Hedgehog; Lys, lysine; P, phosphate group; PKA, protein kinase A; Smo, Smoothened.

active Smo conformations may be found on the cilium when inhibition of Smo ciliary accumulation by Ptch1 is bypassed by small molecules (Rohatgi et al., 2009; Wang et al., 2009; Wilson et al., 2009a). It will be interesting to both test the relationship of various conformational mutants of mouse Smo (for instance, a Smo protein that lacks the Arg clusters) with the cilium, and to investigate the effects of Smo agonists and antagonists on these mutant Smo forms (Zhao et al., 2007).

Disrupted primary cilium formation affects the creation of the Gli3 repressor and Gli activator forms (Liu et al., 2005), yet many important questions concerning the role of the primary cilium in Hh signaling remain unanswered. Initial data in zebrafish suggest that the role of cilia in Hh signaling is conserved among species (Aanstad et al., 2009; Huang and Schier, 2009; Lunt et al., 2009), but it is unclear when cilia were first utilized for Hh signaling in metazoan evolution (Glazer et al., 2010; Rink et al., 2009). Furthermore, the biochemical events on the cilium that affect the processing and activation of full-length Gli proteins are unknown. Finally, the function of the Hh cytosolic signaling complex and its dependence on the primary cilium is largely unexplored, although recent data have shed light on the conservation, constitution and function of this complex, as we discuss below.

The *Drosophila* Hh signaling complex

In *Drosophila*, a cytosolic signaling complex (Table 2) comprising the transcription factor Cubitus interruptus (Ci), the atypical kinesin Cos2, the putative serine/threonine kinase Fu and a sub-stoichiometric amount of the PEST domain protein Su(fu) (for Suppressor of Fused, also called Sufu) is required to transduce the signal from Smo to the nucleus (Aikin et al., 2008; Jia et al., 2005; Zhang et al., 2005). The complex controls the equilibrium between the proteolysis of Ci and the activation of its full-length form, thus providing a mechanism for interpreting graded levels of Hh ligand (Fig. 4A). In the absence of Hh, this complex associates with microtubules (MTs) through Cos2. Cos2 assembles PKA, CKI and GSK3 into a complex that converts Ci into its proteolytically processed repressor form (Ci-75) (Smelkinson et al., 2007; Zhang et al., 2005). Conformational changes in Smo are accompanied by the Cos2-mediated association of the complex with the Smo cytoplasmic tail, which is followed by partial disassembly of the complex (Jia et al., 2003; Liu et al., 2007; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2007; Ruel et al., 2003; Zhao et al., 2007) (Fig. 4B). This disassembly attenuates the limited proteolysis of Ci-155, triggering pathway derepression or activation via the full-length

Table 2. Detailed functions of Hh signaling complex components

Drosophila component	Type of protein	Function in Off pathway state	Function in On pathway state	Zebrafish homolog function	Mouse homolog function	Key references
Cos2	Kinesin (kinesin 4 family), recently shown to be processive on microtubules	Scaffold for PKA, CKI and GSK3, promotes Ci-75 formation, inhibits Ci-155 movement to nucleus	Couples with Smo, promotes rearrangement of signaling complex, facilitating release of Ci, stabilizes and possibly activates Fu	Kif7: Similar function to *Drosophila* in Hh signaling, also may have Hh-independent role in motile cilium function	Kif7: Positive and negative regulator similar to *Drosophila*, regulates Gli2 abundance and Gli3 processing, controls Gli2 and Gli3 trafficking on primary cilium Kif27: Unknown, although associates with Fu	Cheung et al., 2009; Endoh-Yamagami et al., 2009; Farzan et al., 2008; Jia et al., 2003; Lum et al., 2003; Methot and Basler, 2000; Miki et al., 2005; Ogden et al., 2003; Ruel et al., 2003; Tay et al., 2005; Wang and Jiang, 2004; Wilson et al., 2009b; Zhang et al., 2005
Fu	Putative serine-threonine kinase	Promotes Ci-75 formation	Promotes Cos2 and Su(fu) phosphorylation, facilitating release of Ci-155 from complex	Positive effector similar to *Drosophila*, Hh-independent role in motile cilium function	No effect on Hh signaling, Hh-independent role in motile cilium function	Lum et al., 2003; Ruel et al., 2007; Wilson et al., 2009b; Wolff et al., 2003
Su(fu)	Novel protein with PEST domain	Stabilizes Ci-155, prevents nuclear accumulation and/or activation	Stabilizes Ci-155	Inferred to be similar to *Drosophila* Su(fu)	Negative regulator by phenotype, stabilizes Gli proteins, regulates Gli3 nuclear-cytosolic trafficking	Chen et al., 2009; Humke et al., 2010; Kent et al., 2006; Koudijs et al., 2005; Wolff et al., 2003; Zhang, Q. et al., 2006

PKA, protein kinase A; CKI, casein kinase I; GSK3, glycogen synthase kinase 3; Kif27, Kinesin family member 27.

form of Ci (Zhang et al., 2005). Changes to the conformation and composition of the complex, and to its interaction with Smo, provide multiple ways in which to activate the pathway. Table 2 summarizes the mechanistic functions of each component of the *Drosophila* complex, and the behavior of the signaling complex is briefly discussed below.

Hh signaling complex in the absence of ligand

Loss of *cos2* results in the accumulation of Ci-155 and the loss of Ci-75, and Cos2 promotes the limited proteolysis of Ci by directly binding to Ci-155 (Wang et al., 2000; Wang and Jiang, 2004). Cos2 also binds to the kinases PKA, GSK3 and either CKIα or CKIε (Zhang et al., 2005), leading to the hypothesis that Cos2 acts as a scaffold for these kinases to ensure the efficient phosphorylation of Ci-155, thus promoting Ci-75 production (Fig. 4). In support of this notion, the concurrent overexpression of PKA, GSK3 and *Xenopus* CKIε in *cos2* mutant wing discs rescues the Ci processing defect that results from the loss of *cos2* (Zhang et al., 2005). Binding of Cos2 to Ci also tethers the transcription factor in the cytoplasm, preventing its nuclear accumulation (Wang et al., 2000; Wang and Jiang, 2004; Wang and Holmgren, 1999; Wang and Holmgren, 2000). These inhibitory Cos2-Ci complexes are enriched in endosomes through Cos2 binding (Stegman et al., 2004). In the absence of Hh ligand, Fu promotes the efficient processing of Ci (Lefers et al., 2001). Su(fu) weakly associates with the Fu-Cos2-Ci complex, is found in a trimeric complex with Ci and Fu and can bind on its own to the N-terminus of Ci (Lum et al., 2003; Monnier et al., 1998; Stegman et al., 2000) (Fig. 4). The overexpression of Su(fu) inhibits the nuclear accumulation of both full-length Ci-155 and Ci-75 repressor (Lefers et al., 2001; Methot and Basler, 2000), an observation that has been confirmed by the examination of transgenic Ci in *Su(fu)* mutants (Methot and Basler, 2000; Wang et al., 2000). However, the retention of Ci in the cytosol by Su(fu) has only a modest effect on the pathway, and the primary roles for Su(fu) probably lie in the control of Ci stability and nuclear activity (see below).

Hh signaling complex in the presence of ligand

Immunoprecipitation studies have revealed that Smo interacts with Cos2 (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003) to form a signaling complex that increases in quantity after Hh stimulation and that Cos2 localizes to the plasma membrane after its association with Smo. Interestingly, Smo binds to the same region of Cos2 as do PKA, GSK3 and CKIα/ε, and thus Smo might compete with these kinases to bind to Smo (Jia et al., 2003; Lum et al., 2003) (Fig. 4B). Hh signaling causes a slight Fu-dependent destabilization of Cos2 (Liu et al., 2007; Ruel et al., 2003). Fu-dependent phosphorylation of Cos2 on Ser572 might also weaken the Cos2-Ci association, promoting the release of Ci from the complex (Ruel et al., 2007). Thus, Cos2 might promote pathway activation by its increased binding or by altering its association with membrane-proximal and cytosolic regions of Smo, eliminating a stable Cos2-kinase scaffold and inhibiting the efficient processing of Ci-155. Cos2 preferentially binds phosphorylated Smo (Lum et al., 2003), perhaps reflecting that the phosphorylation of Smo might expose different Cos2-interacting surfaces, although its phosphoresidues are not predicted to be part of the interacting

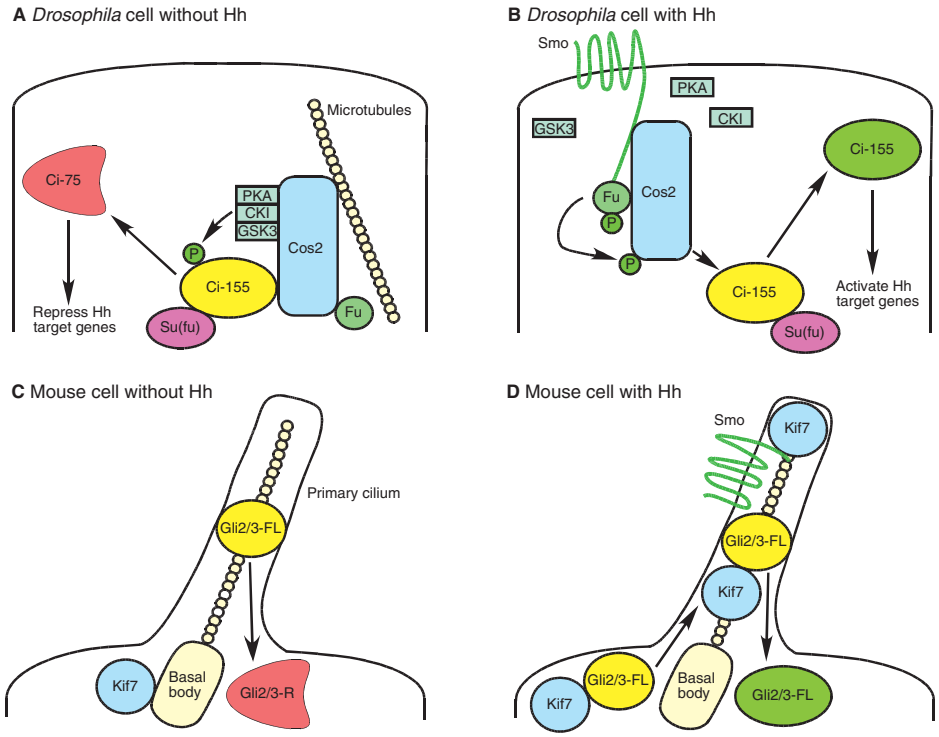

Fig. 4. Hedgehog signaling complex function in *Drosophila* and mouse. Modes of action of the Hh signaling complex. Matching colors denote homologs. (**A**) In *Drosophila*, Cos2 associates with microtubules and scaffolds PKA, CKI and GSK3. This leads to efficient phosphorylation of Ci-155 and limited proteolysis to generate Ci-75. (**B**) Cos2 binds to the C-tail of Smo when Smo is in its active conformation, leading to the dissociation of PKA, CKI and GSK3. Fu is phosphorylated and in turn phosphorylates Cos2, which might promote the dissociation of Ci-155, leading to activation of Hh target genes. Removal of components of the Hh signaling complex has variable effects on Ci-155 and Ci-75 levels, and the relationship of these protein levels to the activation or repression of Hh target genes is not completely understood. (**C**) In the mouse, Gli2 and Gli3 are present in small amounts on the primary cilium in the absence of Hh ligand, and the cilium is required for generation of Gli repressors. Kif7 is located at the base of the cilium. (**D**) Kif7 binds Gli proteins and regulates their translocation to the primary cilium after stimulation of a cell with Hh ligand. Cilia promote activation of Gli2 and Gli3 through unknown mechanisms. Kif7 might also bind Smo, and Smo is required for Kif7 to move up the cilia. The precise makeup of the vertebrate complex in the absence and presence of Hh remains to be determined, although genetic evidence demonstrates that Fu is not an essential part of it. Ci, Cubitus interruptus; CKI, casein kinase I; Cos2, Costal2; FL, full-length; Fu, Fused; GSK3, glycogen synthase kinase 3; Hh, Hedgehog; Kif7, kinesin family member 7; P, phosphate group; PKA, protein kinase A; R, repressor; Smo, Smoothened; Su(fu), Suppressor of fused.

surface. A net increase in the ratio of active to inactive Smo would shift the balance of active to repressive complexes, leading to Ci-155 release and to pathway activation. Cos2 also stabilizes Fu and promotes its phosphorylation (see Table 2), which could play an important role in promoting Fu kinase activity (Claret et al., 2007; Lum et al., 2003; Robbins et al., 1997; Ruel et al., 2003; Sisson et al., 1997). Fu might also act more directly in Ci activation, either upstream, by promoting Smo phosphorylation (Liu et al., 2007; Lum et al., 2003), or downstream, by trafficking Ci to an as-yet-unidentified binding partner for activation. Furthermore, Fu can stimulate Hh pathway activation by antagonizing Su(fu) and promoting its phosphorylation; loss of *Su(fu)* also strongly suppresses the *fu* mutant phenotype, indicating that an antagonistic relationship exists between these two gene products (Lum et al., 2003; Préat, 1992).

The observation that Ci protein levels are reduced in *Su(fu)* mutants yet its loss results in no obvious phenotype is perplexing (Lefers et al., 2001; Ohlmeyer and Kalderon, 1998; Préat, 1992). Initially, it was proposed that Su(fu) opposes the formation of a labile, hyperactive form of Ci (Ohlmeyer and Kalderon, 1998). There are many examples of transcription factors whose activities are controlled by the ubiquitin-proteasome system (Kodadek et al., 2006) and, in some instances, ubiquitination is required for the full activation of proteins, such as with Myc (Muratani and Tansey, 2003). Identifying that the proteasome has a role in activating Ci is challenging given its essential function in the processing of Ci-155 to Ci-75. Ultimately, a definitive relationship between reduced Ci levels and pathway activation remains to be biochemically demonstrated, as does the relationship between Ci-155 levels and the hypothetical Ci activator form. An alternative explanation for the reduction of Ci protein levels in a *Su(fu)* background is its increased degradation by factors such as HIB (Hedgehog-induced MATH and BTB domain-containing protein; also known as *roadkill*) (Kent et al., 2006; Zhang, Q. et al., 2006). HIB is a member of the MATH (Meprin and Traf homology)-BTB (Broad complex, Tramtrack and Bric a Brac) protein family, which are adapters for the Cullin-3-based ubiquitin ligases, and its overexpression results in the degradation of full-length Ci protein (Kent et al., 2006; Zhang, Q. et al., 2006). HIB is inhibited by Su(fu) in a dose-dependent manner (Zhang, Q. et al., 2006) (Fig. 5A), and HIB and Su(fu) compete for binding to Ci (Zhang, Q. et al., 2006). Thus, the removal of Su(fu) results in an increased turnover of full-length Ci (Zhang, Q. et al., 2006) (Fig. 5A). Interestingly, *HIB* is upregulated in response to Hh signaling, so it

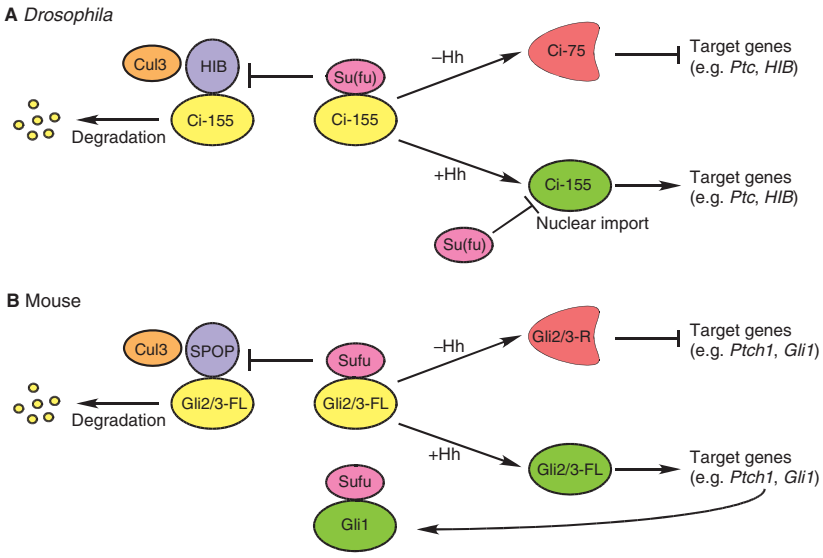

Fig. 5. Sufu and HIB/SPOP action on Ci/Gli proteins. A model of how Sufu protects Ci/Gli proteins from HIB/SPOP-promoted degradation. (**A**) Fly Su(fu) binds Ci-155 and competitively inhibits HIB binding, which recruits the Cul3 E3 ligase and completely degrades Ci. Depending on the state of Hh pathway activation, Ci is either processed into the Ci-75 repressor after dissociation from Su(fu) or activates transcription of Hh target genes such as *Ptc* and *HIB*. Su(fu) also impedes nuclear accumulation of Ci-155. (**B**) Mouse Sufu binds to Gli2 and Gli3, protecting them from SPOP-mediated degradation. As in *Drosophila*, Gli2 and Gli3 are either proteolytically processed into repressors or activate Hh target genes, including *Ptch1* and *Gli1*. SPOP is unable to bind Gli1 and does not cause its degradation. Sufu does not seem to have a profound effect on endogenous Gli1, Gli2 or Gli3 nuclear-cytoplasmic shuttling. Ci, Cubitus interruptus; Cul3, Cullin3; FL, full-length; Hh, Hedgehog; HIB, Hedgehog-induced MATH and BTB domain-containing protein; Ptc/Ptch1, Patched; R, repressor; SPOP, Speckle-type POZ protein; Su(fu)/Sufu, Suppressor of fused.

might participate in a feedback loop that limits Ci activity after pathway activation (Kent et al., 2006; Zhang, Q. et al., 2006). Low levels of *HIB* might be sufficient for an increased rate of Ci turnover in the absence of Su(fu). However, the transcriptional activity of Ci-155 and Ci-75 appears to be unaffected in the absence of Su(fu), potentially explaining the lack of phenotype observed in *Su(fu)* mutants (Préat, 1992). Furthermore, the dominant role of Cos2 in sequestering Ci-155 in the cytosol might protect Ci-155 from HIB-promoted degradation.

The *Drosophila* Hh signaling complex displays complicated behavior and variable biochemical composition depending on the state of pathway activation, yet a clearer picture has emerged in recent years. The central role of a Cos2 scaffold, which relays conformational changes in Smo to Ci/Gli proteins, appears to be evolutionarily conserved, yet associated effectors in vertebrates remain elusive. In the following section, we discuss the current understanding of vertebrate homologs of key *Drosophila* Hh signaling complex components.

The vertebrate Hh signaling complex

The lack of extensive duplication of cytosolic Hh effectors in vertebrates has facilitated targeted knockout studies in mice and morpholino knockdowns in zebrafish. Strikingly, recent data indicate that the function of vertebrate Cos2 and Sufu homologs is similar to their *Drosophila* counterparts, yet some vertebrate Hh components require the primary cilium (Table 2). Subfunctionalization after gene duplication is a probable explanation of the divergent functions of Fu and also the differences in Hh pathway mutant phenotypes among diverse species.

Vertebrate Cos2 orthologs

Sequence analysis of kinesin-like proteins in vertebrate genomes has revealed the existence of two putative orthologs of Cos2: Kif7 in zebrafish and Kif7 and Kif27 in mice (Katoh and Katoh, 2004a; Katoh and Katoh, 2004b; Tay et al., 2005). In zebrafish, Kif7 behaves similarly to *Drosophila* Cos2, as a predominantly negative regulator of Hh target genes. It also physically interacts with Gli1, a Ci homolog, suggesting it might indeed function as a cytosolic scaffold in this system (Tay et al., 2005). Kif7 also plays an Hh-independent role in the establishment of left-right asymmetry (Wilson et al., 2009b), a process that does not rely on Smo function in zebrafish (Chen et al., 2001).

Initial studies of *Kif7* and *Kif27* in NIH 3T3 cells, a mouse fibroblast cell line responsive to Hh ligands, demonstrated that no substantial perturbation of Gli reporter activity occurs when these transcripts were knocked down using RNAi (Varjosalo et al., 2006). However, the genetic disruption of *Kif7* in mice results in significant Hh-related phenotypes (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). In the embryonic limb of *Kif7*-null mice, for example, polydactyly is evident, which is indicative of disrupted Gli3 repressor (Gli3R) function. Similarly, in the ventral neural tube of *Kif7*-null mice, the motoneuron progenitor population is expanded, indicating the loss of Gli3R activity in this tissue as well. When *Kif7* mutants are crossed onto *Gli2*- and *Ptch1*-null backgrounds, the resulting mutant phenotypes reveal a positive role for Kif7 in mammalian Hh signaling. Neural tube ventralization seen in *Ptch1*-null embryos, which is due to maximal Hh pathway activation, is reduced in *Ptch1; Kif7* double mutants, and floor plate induction (which requires Gli activator function) is defective in *Kif7* mutants when one copy of Gli2 is concomitantly removed. The role for Cos2 as a switch downstream of Smo is therefore evolutionarily conserved, and Cos2/Kif7 is probably a core ancestral component of the Hh pathway that has both positive and negative roles.

Kif7 function in mammals depends on the primary cilium, as single *Ift172* and compound *Ift172; Kif7* mutant mouse embryos are phenotypically indistinguishable (Liem et al., 2009). Kif7-GFP fusion proteins localize to the base of the cilium and the proportion of Kif7-GFP at the cilium tip increases after Hh stimulation (Endoh-Yamagami et al., 2009; Liem et al., 2009) (Fig. 4C,D). Smo might bind to Kif7 in mice but it is required for the accumulation of Kif7 in cilia and, in turn, Kif7 promotes an increase in Gli2 and Gli3 protein levels in the cilium (Endoh-Yamagami et al., 2009) (Fig. 4D). Kif7 is also required for regulating Gli2 abundance and for efficient Gli3 proteolysis, as is evident in *Kif7*-null embryos, which have increased levels of full-length Gli2 and Gli3 (Cheung et al., 2009; Endoh-Yamagami et al., 2009). Thus, Kif7 probably serves as a scaffold for the production of Gli repressors, which might occur at the base of the primary

cilium. Trafficking of Kif7 on the ciliary axoneme in response to Hh might allow its binding to Smo and the activation of full-length Gli2 and Gli3.

Divergent roles of vertebrate Fused

Previous in vitro findings had indicated that mammalian Fu has a weak role in potentiating Gli activator function and in opposing Sufu (Murone et al., 2000). In zebrafish, morpholino knockdown of *fu* results in mild Hh somitic phenotypes (Wolff et al., 2003); stronger Hh phenotypes, including cyclopia, are seen when *fu* and *p53* morpholinos are co-injected (Wilson et al., 2009b). As in *Drosophila*, *fu* is epistatic to other Hh pathway genes (Wolff et al., 2003). Surprisingly, the targeted disruption of the single mouse *Fu* ortholog had no effect on mouse embryonic patterning (Chen et al., 2005; Merchant et al., 2005). *Fu*-null mutants die after birth and have an Hh-independent defect in the central pair of microtubules of motile cilia (Wilson et al., 2009a). Whether loss of Fu activity is compensated for in vertebrate Hh signaling is unclear. It is also not known if other kinases such as Cdc2l1 (Cdk11b – Mouse Genome Informatics) or Ulk3 have replaced Fu in the mammalian Hh pathway, although an investigation of whether these kinases bind and phosphorylate Kif7 is warranted given their positive effects on Gli activity in vitro (Evangelista et al., 2008; Maloverjan et al., 2010; Varjosalo et al., 2008). In zebrafish, *fu* morphant phenotypes have revealed an Hh-independent role for *fu* in the biogenesis of motile cilia (Wilson et al., 2009a). In addition to Hh patterning defects, *fu* morphants exhibit defects in left-right asymmetry, which are not observed in *smo* morphants (Chen et al., 2001; Wilson et al., 2009b). Thus, in zebrafish, components of the Hh signaling complex are also utilized in an unrelated cellular process. Intriguingly, mouse Kif27, but not Kif7, physically interacts with mouse Fu and localizes to the basal body of motile cilia (Wilson et al., 2009b), suggesting that Kif27 and Fu partner to control central pair microtubule assembly in motile cilia. Understanding how Fu evolved divergent functions in distinct cellular processes will yield important insight into the origin of the Hh pathway.

Sufu regulates vertebrate Hh transduction

Mammalian Sufu binds all three Gli proteins and can prevent Gli1, Gli2 or Gli3 from entering the nucleus when overexpressed in vitro (Ding et al., 1999; Kogerman et al., 1999). In sharp contrast to fly *Su(fu)* mutants, targeted disruption of mouse *Sufu* results in a drastic upregulation of the Hh pathway and lethality by embryonic day (E) 9.5 (Cooper et al., 2005; Svärd et al., 2006). Knockdown or genetic ablation of *Sufu* in NIH 3T3 cells or in MEFs results in ligand-independent activation of Gli reporters (Svärd et al., 2006; Varjosalo et al., 2006). In addition, morpholino knockdown of *sufu* in zebrafish results in a weak gain-of-function Hh phenotype in the myotome (Wolff et al., 2003). However, mouse *Sufu; Fu* double mutants phenocopy *Sufu* mutants (Chen et al., 2009). The altered role of Fu in vertebrate Hh transduction (Chen et al., 2005; Merchant et al., 2005; Wilson et al., 2009b) suggests that the cytoplasmic regulatory circuitry has changed to a point where the *Sufu* and *Fu* gene products no longer antagonize one another. In contrast to *Drosophila* Su(fu), mouse Sufu does not appear to be essential for cytosolic retention of overexpressed eGFP-Gli1 but might regulate the nuclear-cytoplasmic distribution of endogenous Gli2 and Gli3 (Chen et al., 2009; Humke et al., 2010; Svärd et al., 2006) (Fig. 5B). Yeast two-hybrid screens identified the mSin3a-SAP18 core-repressor complex as a potential binding partner of mouse Sufu (Cheng and Bishop, 2002; Paces-Fessy et al., 2004), indicating a potential nuclear role for Sufu in the assembly of transcriptional repression complexes. Studies using a synthetic multimerized Gli-luciferase transcriptional reporter have indicated that Sufu and SAP18 synergistically repress Gli-dependent transcription in HEK 293T cells (Cheng and Bishop, 2002) but this result has thus far not been replicated, either in other cell lines or in vivo (Chen et al., 2009).

Loss of *Sufu* in mammals drastically reduces the levels of Gli2 and Gli3 protein (Chen et al., 2009). However, despite its ciliary localization, the effect of Sufu on Gli2 and Gli3 stability is independent of the primary cilium (Chen et al., 2009; Jia et al., 2009). The role for Sufu in controlling Ci/Gli stability is evolutionarily conserved, as the antagonism between Sufu and Spop (Speckle-type POZ protein) (Zhuang et al., 2009), a mouse homolog of *Drosophila* HIB, is maintained in mammalian cell culture (Chen et al., 2009; Zhang et al., 2009; Zhang, Q. et al., 2006) (Fig. 5). *Gli1* might be a major contributor to the *Sufu* phenotype because its expression is upregulated in a $Sufu^{-/-}$ background, it is refractory to Spop-promoted degradation and RNAi of *Gli1* in $Sufu^{-/-}$ MEFs significantly reduces pathway activity (Chen et al., 2009; Svärd et al., 2006) (Fig. 5B). It is also possible that loss of Gli repressors and/or gain of Gli activators (Humke et al., 2010) in the absence of Sufu could add to Hh pathway activation. The duplication of the ancestral *Ci* gene, coupled with the subfunctionalization of the mammalian Gli proteins (partitioning their differential activity and regulation) and the formation of novel transcriptional feedback loops (as discussed further below), might together explain why the conserved action of Sufu has different net effects in flies and mice.

Modifications in Ci/Gli regulation

In *Drosophila*, Ci provides all known Hh-dependent transcriptional activation and repression functions, and so regulation of its proteolysis or activation is crucial (Methot and Basler, 2001). General and lineage-specific duplication of *Ci* has resulted in partitioning of its activator and repressor functions among several *Gli* genes in vertebrates (Bai et al., 2002; Bai and Joyner, 2001; Bai et al., 2004; Chen, Y. et al., 2004; Dai et al., 1999; Hui et al., 1994; Karlstrom et al., 1999; Karlstrom et al., 2003; Ke et al., 2005; Ke et al., 2008; Matise et al., 1998; Park et al., 2000; Sasaki et al., 1999; Tyurina et al., 2005) (Table 1).

Several factors complicate the dissection of the precise role of individual Gli factors in the Hh response. First, redundancy in Gli activator and repressor function precludes the attribution of specific phenotypic outcomes to a single *Gli* gene (Bai et al., 2002; Bai and Joyner, 2001; Motoyama et al., 1998; Motoyama et al., 2003). Second, the evolution of *Gli* transcriptional feedback loops in vertebrates has added robustness and additional layers of complexity to the Hh-dependent transcriptional network. A major factor in this might be the regulation of *Gli1* by both the Gli3 repressor and Gli2 activator, as shown by in situ analysis and, more recently, by chromatin immunoprecipitation (Hu et al., 2006; Lee et al., 1997; Marigo et al., 1996b; Motoyama et al., 2003; Vokes et al., 2008). Removal of the Gli3 repressor, either genetically or through the modulation of factors that control its stability, might result in derepression or even activation at the *Gli1* locus. Third, the post-transcriptional regulation of the Gli proteins is more complex, as changes in ancestral Ci domain architecture in the individual Gli proteins have led to alterations in the regulation of specific Gli proteins by limited or complete proteolysis or destruction (reviewed in Jiang and Hui, 2008).

The stability of Ci is regulated at multiple levels by E3 ubiquitin ligases (Dai et al., 2003; Jiang and Struhl, 1998; Lee et al., 2002; Zhang, Q. et al., 2006). Similar to Ci, all three Gli proteins have several signals for limited or complete proteolysis. Notably, degradation sequences (termed degrons) are found in Gli1 and Gli2 for binding to β-TrCP, an E3 adapter protein (Bhatia et al., 2006; Huntzicker et al., 2006; Pan et al., 2006). These degrons are utilized differentially, as they are required for the destruction of Gli1, for either the processing or destruction of Gli2, and for the processing of Gli3 (Bhatia et al., 2006; Huntzicker et al., 2006; Pan et al., 2006). Additional degrons are present in Gli1, which might utilize the Numb-Itch ubiquitination pathway or some other unidentified mechanisms of degradation (Di Marcotullio et al., 2006; Huntzicker et al., 2006). Further studies are needed to resolve how these multiple degradative pathways are utilized to control the availability of full-length and repressor forms of the Gli proteins and whether this enhances the range of Hh response. It is not known how a cell discriminates between specific degrons within a Gli. Finally, whether Sufu is a general protective factor or specifically antagonizes Spop-mediated degradation of Gli2 and Gli3 remains to be investigated.

Conclusions and outstanding questions

As a result of recent progress in elucidating the roles of vertebrate Kif7, Fu and Sufu in cytosolic Hh signaling and Hh-independent processes, new areas of investigation have opened up. The mechanism of Smo regulation by Ptch and the involvement of small molecules such as oxysterols has been summarized elsewhere (Rohatgi and Scott, 2007). Below, we focus on unanswered cell biological, biochemical and transcriptional questions relating to the primary cilium and cytosolic Hh components and speculate on possible routes of Hh pathway evolution.

Cytosolic transduction of Hh in mammals

Consistent with Hh transduction in *Drosophila*, mammalian Hh signaling utilizes a kinesin scaffold to interact with Smo and to control Ci/Gli proteolysis (Cheung et al., 2009; Endoh-Yamagami et al., 2009; Liem et al., 2009). The increased amounts of Smo, Kif7, Gli2 and Gli3 on the primary cilium suggest that a signaling complex undergoes assembly or rearrangement in response to Hh signaling. Similarly, the requirement of Kif7 for efficient Gli3 proteolysis implies that it might also be a scaffold for PKA, GSK3 and CKI. It remains to be seen whether Kif7 is a processive ciliary motor or relies on direct physical interaction with Smo for cilium movement. In this instance, trafficking of a Smo-Kif7 complex could be mediated by β-arrestin-bridging such a complex to the Kif3 motor (Kovacs et al., 2008). Detailed real-time trafficking studies and the biochemical assessment of the assembly and disassembly of the signaling complex will be needed to further dissect these questions.

Smo physically resembles a G-protein-coupled receptor (GPCR), yet there are conflicting data as to whether coupling to a Gα subunit activates Hh-dependent transcriptional responses. In cultured *Xenopus* melanophores, insect cells and mammalian tissue culture, Smo stimulates Gαi-dependent responses and GTP binding to Gαi proteins (DeCamp et al., 2000; Riobo et al., 2006). Despite this, no in vivo effect on Hh signaling has been observed upon activation or inhibition of Gαi in vertebrates (Low et al., 2008). Recent data have shown that genetic manipulation of Gαi in *Drosophila* affects Hh signaling, primarily through classical effects of Gαi on PKA activity (Ogden et al., 2008). Surprisingly, Gαi interacts with Cos2 in an Hh-dependent fashion but no physical association with Smo was reported (Ogden et al., 2008). A common role in *Drosophila* and mouse Hh signaling for G-protein-receptor-coupled kinase 2 (GRK2) has been demonstrated, indicating that a link exists between classical GPCR machinery and Smo activation (Chen, W. et al., 2004; Meloni et al., 2006; Philipp et al., 2008). GRK2/GPRK2 and β-arrestins might influence the membrane trafficking of Smo, thus affecting pathway activity through controlling the ability of Smo to reach the cell surface in flies or the cilium in vertebrates (Chen, W. et al., 2004; Kovacs et al., 2008; Molnar et al., 2007). β-arrestins could also facilitate the coupling of Smo to downstream components such as Kif7, consistent with their more recently discovered role as signaling scaffolds (Lefkowitz and Shenoy, 2005).

Vertebrate Hh modulators: regulators of primary cilium function?

Several genes that play unique roles in vertebrate Hh transduction have been identified, such as sil, tectonic, FK506 binding protein 8 (FKBP8), talpid 3 and iguana (dzip1) (Bulgakov et al., 2004; Davey et al., 2006; Izraeli et al., 2001; Reiter and Skarnes, 2006; Sekimizu et al., 2004; Wolff et al., 2004). Many of these genes, such as tectonic, which encodes a protein of unknown function, affect both Gli activator and repressor function and affect the pathway downstream of Ptch1 and Smo (Reiter and Skarnes, 2006). This is similar to the talpid 3 and iguana genes, which were recently shown to control ciliogenesis (Glazer et al., 2010; Rink et al., 2009; Yin et al., 2009). Further investigation is needed to determine whether the remaining vertebrate-specific genes act in a similar fashion. Genetic studies of the vesicle transport protein and GTPase Rab23, a cell-autonomous negative regulator of vertebrate Hh signaling, showed that Rab23 controls Gli2 and Gli3 activity (Eggenschwiler et al., 2006). Rab23 could regulate the trafficking of Hh pathway components that inhibit Gli activator function. By contrast, the GTPase Arl13b appears to control Gli activator production and sequestration as Gli activators are constitutively active (albeit at low levels) in the absence of Arl13b (Caspary et al., 2007). These, and other Rab proteins involved in the biogenesis of the primary cilium (Yoshimura et al., 2007), are likely to be useful targets for investigating the dynamics of Smo and Gli movement within the cell and on the primary cilium, and their relationships to states of pathway activation (Oro, 2007).

Mechanism of Ci/Gli action on target enhancers and promoters

A large gap in our understanding of Gli-dependent transcription stems from a dearth of information regarding the mechanism of action of Gli proteins on endogenous enhancers and promoters. It is unclear how combinations of Ci/Gli activators and repressors within a given cell are utilized to produce a specific transcriptional response. A number of putative co-activators (including CBP, mediator and Hoxd12) and co-repressors (e.g. Sap18, mSin3a, and Ski) have been identified, although these effects and interactions have not been observed in an endogenous context (Akimaru et al., 1997; Chen, Y. et al., 2004; Cheng and Bishop, 2002; Dai et al., 2002; Zhou et al., 2006). Recent work using chromatin immunoprecipitation of an artificially tagged mouse Gli1 activator and Gli3 repressor has allowed the identification of several bona fide endogenous Gli binding sites (Vokes et al., 2007; Vokes et al., 2008). Further studies of the biochemical mechanism of Gli factors at these newly identified loci should shed light on a number of unanswered questions. One such question is whether Gli activator and repressor forms act at the same Gli binding site, although the

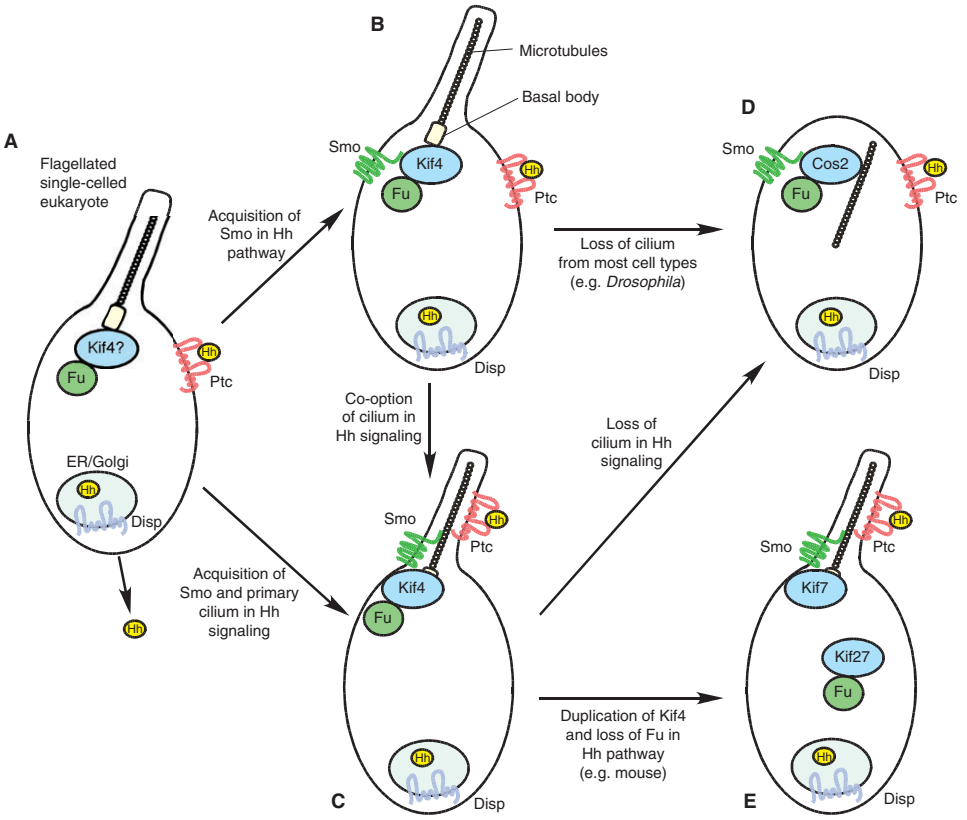

Fig. 6. Possible routes of Hh pathway evolution. A model of Hh pathway evolution. (**A**) Single-celled eukaryotes (for example, the collared flagellate *Monosiga brevicollis*) have *Disp*, *Ptc*, *Hh* and *Fu* genes. An ancient export-import system of *Hh*, *Ptc* and *Disp* could have been present in this organism (Hausmann et al., 2009), and a Fu-kinesin 4 (Kif4) complex might have been required for the assembly of the 9+2 cilium/flagellum. (**B**,**C**) Smo is incorporated into a regulatory circuit with Ptc, either prior to the involvement of Smo and Ptc function with the cilium (B) or concomitantly (C). In both scenarios, the Fu-kinesin 4 complex is recruited to function with Smo. (**D**) In flies, the primary cilium is not utilized for Hh signaling. It is currently unclear whether cilia represent an ancestral state for Hh transduction and flies have 'rewired' the pathway, or if cilia were incorporated into the pathway after divergence of arthropod and chordate lineages. (**E**) Gene duplication of Kif4 (subsequently generating Kif7 and 27) in tetrapod lineages has led to loss of essential Fu function in mammalian Hh signaling but Kif7 is retained. It is not known if an unrelated kinase compensates for the loss of Fu. Cos2, Costal2; Disp, Dispatched; ER, endoplasmic reticulum; Fu, Fused; GSK3, glycogen synthase kinase 3; Hh, Hedgehog; Kif4, kinesin family member 4; Kif7, kinesin family member 7; Kif27, kinesin family member 27; Ptc, Patched; Smo, Smoothened; Su(fu), Suppressor of fused.

data suggest that they might for a subset of genes expressed both in neural tissue and the limb mesenchyme (Vokes et al., 2007; Vokes et al., 2008). This issue is of importance because many of the transcriptional mechanisms inferred from developmental studies of Hh pathway components rely on the assumption that Gli activator and repressor forms act on the same binding sites. Another question concerns a comparison of the modes of transcriptional activation and repression and the cofactors required for different classes of Hh target genes. The expression of some Hh target genes depends on pathway activation (e.g. that of *Gli1*), whereas other targets must be expressed prior to the induction of Gli activators, which then have their expression increased via positive feedback (e.g. *Ptch1*). Thus, it will be interesting to discover possible similarities and differences in the transcription of different types of Hh target genes.

Hh and Wnt signaling

Similarities in Hh and Wnt transduction have been described, and typically advances in understanding of one cascade have led to similar conceptual breakthroughs in the other (reviewed in Kalderon, 2002; Nusse, 2003). The discovery that primary cilia play a key role in Hh signaling led to the speculation that this organelle is involved in Wnt transduction. Genes such as inversin and the Bardet-Biedl syndrome (BBS) family, which are essential for proper basal body structure and function, can modulate planar cell polarity (PCP) in a tissue-specific manner (Gerdes et al., 2007; Ross et al., 2005; Simons et al., 2005). However, there are conflicting reports concerning the role of cilia and IFT in canonical Wnt signaling (Corbit et al., 2008; Ocbina et al., 2009). Mice and zebrafish deficient in IFT genes lack overt Wnt phenotypes, such as defects in gastrulation, and exhibit morphological abnormalities apparently only from misregulated Hh transduction (Eggenschwiler and Anderson, 2007; Huang and Schier, 2009). Thus, cilia are not essential for canonical Wnt transduction in early embryonic development, although Wnt signaling might utilize cilia later in gestation or during postnatal development. Further dissection of the extent of convergent evolution of Hh and Wnt signaling in different species will illuminate the general design of such signaling pathways.

Evolution of Hh signaling

The recent discoveries of the role of the primary cilium in vertebrate Hh transduction, as well as evidence indicating that Fu is not essential in mice, raises several questions concerning the

origins and evolution of the Hh pathway. One issue is whether the utilization of the primary cilium in Hh signaling reflects an ancient role for the organelle or whether the cilium has been incorporated into the molecular circuitry of a pre-existing Hh architecture (Fig. 6). Disruption of cilia in planaria does not recapitulate Hh knockdown phenotypes, yet this does not preclude cilia from being an integral part of ancestral Hh signaling (Glazer et al., 2010; Rink et al., 2009). Additional studies of Hh and cilia function in other metazoan model organisms will provide further insight to this question.

Genes containing a Fu kinase domain are easily identifiable in all branches of the eukarya, with the exception of fungi (our unpublished results). This includes plants and single-celled eukaryotes such as *Chlamydomonas reinhardtii*. Every identified role of the Fu kinase family in eukaryotes involves microtubules and/or some aspect of cell polarity, even in organisms that lack cilia (Oh et al., 2005; Tang et al., 2008; Wilson et al., 2009b). Thus, Fu and ancestral Cos2/Kif7 might function in a basic cilium structural or polarity pathway that has been co-opted by the Hh pathway in organisms such as *Drosophila* or zebrafish (Fig. 6). The fact that mouse Fu rescues Hh-dependent and independent phenotypes in zebrafish *fu* morphants implies that the underlying mode of Fu action is similar for two seemingly unrelated processes. An ancient role for Fu and Cos2/Kif7 orthologs in ciliogenesis is supported by a recent study in planaria, in which the RNAi knockdown of their transcripts disrupted cilium function but not Hh signaling (Rink et al., 2009). The future examination of the role of the Fu kinase in single-celled eukaryotes such as *Chlamydomonas*, which are bi-flagellated yet lack an intact Hh pathway, will shed further light on the ancestral mechanistic function of Fu and Cos2/Kif7. Furthermore, the data suggest that changes in the subcellular localization or cell-type-specific expression of signaling components is a mechanism for pathway evolution and this will be useful in assessing the evolution of other signal transduction cascades.

The assembly of Hh pathway components into an ordered signaling pathway during evolution is a poorly understood process, yet recent genome surveys and functional studies have clarified a possible order of events (Fig. 6). The choanoflagellate *Monosiga brevicollis* contains orthologs of *Hh*, *Ptc*, *Disp* and *Fu*, yet lacks a recognizable *Smo* homolog (King et al., 2008). The basic principles of Disp-mediated release of Hh, and subsequent binding to Ptc, might have thus been established in the last common ancestor of choanoflagellates and metazoans (Hausmann et al., 2009). Acquisition of Smo, co-option of Fu and recruitment of the cilium or the Hedgehog signaling complex in signaling might have subsequently occurred after the split of these two lineages (Fig. 6). Comparative studies of chordates, invertebrates and flagellated single-cell eukaryotes provide a unique and exciting opportunity to test mechanistic theories of Hh pathway construction and serve as a paradigm of pathway evolution.

In summary, the past several years have seen significant progress in our understanding of the molecular mechanism and evolution of the Hh transduction cascade. We anticipate that the next decade will yield more mechanistic insight that will further illuminate the conserved and divergent aspects of Hh signaling at the membrane and in the cytoplasm, as well as providing new insights into how this fascinating pathway was assembled in ancestral eukaryotes and subsequently adapted in different evolutionary lineages. Attaining a thorough understanding of Hh signaling is of vital importance for developing a mechanistic understanding of congenital anomalies and disease, and this line of research continues to hold great promise for developing rational therapies for Hh-associated disorders.

Acknowledgements

We thank Kaveh Ashrafi, David Casso, Jau-Nian Chen, Phillip Dumesic and Ross Metzger for critical comments on the manuscript, which was improved by helpful critiques from the reviewers and editor. We sincerely apologize to those whose original articles were not cited owing to space constraints. P.-T.C. is funded by the NIH. Deposited in PMC for release after 12 months.

Competing interests statement

The authors declare no competing financial interests.

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