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REVIEW

Hedgehog signaling and the primary cilium: implications for spatial and temporal constraints on signaling

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ABSTRACT

The mechanisms of vertebrate Hedgehog signaling are linked to the biology of the primary cilium, an antenna-like organelle that projects from the surface of most vertebrate cell types. Although the advantages of restricting signal transduction to cilia are often noted, the constraints imposed are less frequently considered, and yet they are central to how Hedgehog signaling operates in developing tissues. In this Review, we synthesize current understanding of Hedgehog signal transduction, ligand secretion and transport, and cilia dynamics to explore the temporal and spatial constraints imposed by the primary cilium on Hedgehog signaling *in vivo*.

KEY WORDS: Hedgehog, Cell cycle, Cilia, Signaling

Introduction

The primary cilium is a microtubule-based organelle that acts as a cellular antenna. Concentrating membrane receptors and signaling components, the primary cilium projects into the signal-rich extracellular environment and transduces signals to influence cell fate (Anvarian et al., 2019). Defects in primary cilium structure and function can lead to a spectrum of developmental disorders, many of which are rooted in disrupted signaling (Reiter and Leroux, 2017). Of the signaling pathways associated with the primary cilium, vertebrate Hedgehog (Hh) signaling is the best characterized with respect to its relationship to the cilium (Bangs and Anderson, 2016). Hh signaling was originally identified for its patterning role in *Drosophila* (Nüsslein-Volhard and Wieschaus, 1980) and has important functions in the growth and morphogenesis of many vertebrate tissues, notably the nervous system, limb and skeleton (Chiang et al., 1996; Echelard et al., 1993; Riddle et al., 1993). Hh operates as a mitogen, driving proliferation and tissue growth, or as a morphogen, instructing cell fate, often through concentration gradients of secreted Hh ligands (Dessaud et al., 2008; Ericson et al., 1997; Wechsler-Reya and Scott, 1999). The biochemical mechanisms of vertebrate Hh signaling have been recently reviewed (Kong et al., 2019). Briefly, in the absence of Hh ligand, the Hh receptor patched 1 (Ptch1) inhibits the activator smoothened (Smo), keeping the pathway in its off state (Fig. 1A). Binding of one of the three vertebrate Hh ligands (Sonic hedgehog, Shh; Indian hedgehog, Ihh; or Desert hedgehog, Dhh) to Ptch1 releases the inhibition of Smo and allows downstream pathway activation mediated by the activator forms of Gli transcription factors.

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In vertebrates, Hh signaling requires the primary cilium. A primary cilium consists of nine microtubule doublets that extend from the membrane-docked mature centriole (termed a 'basal body', once docked) to form an axoneme. The axoneme is surrounded by a ciliary membrane, which is a specialized and distinct compartment of the plasma membrane. Transport in and out of the cilium, which is necessary for ciliogenesis, maintenance and signaling, occurs bi-directionally along microtubules via the process of intraflagellar transport (Ishikawa and Marshall, 2017; Kozminski et al., 1993). The discovery that mutations in intraflagellar transport pathway genes cause defects in Hh signaling provided the first evidence for the role of primary cilia in Hh signaling (Huangfu et al., 2003).

Much subsequent work has identified three distinct aspects of vertebrate Hh signaling that occur in cilia (Fig. 1B). First, cilia may be important for initial signal reception, as the Ptch1 receptor concentrates in the ciliary membrane (Rohatgi et al., 2007). Second, cilia are important for pathway activation, as the repression of Smo by Ptch1 occurs in cilia, and Smo accumulates in the ciliary membrane when signaling is activated (Corbit et al., 2005; Rohatgi et al., 2007). Finally, cilia are required for transcription factor processing, as the Gli2 and Gli3 transcription factors travel via intraflagellar transport to the tips of cilia and are processed into their activator or repressor forms (Haycraft et al., 2005; Huangfu and Anderson, 2005; Liu et al., 2005).

Although primary cilia have traditionally been studied in quiescent cells, recent advances have provided insight into the temporal dynamics and spatial localization of cilia in developing tissues. It has thus become clear that our understanding of the Hh pathway must expand to reflect the unique cell biology of this organelle. In this Review, we synthesize recent work about the mechanisms of Hh signal transduction, the dynamics of cilia in cycling cells, and the signal transport and reception of Hh ligand to highlight how reliance on a cilium imposes spatial and temporal constraints on Hh signaling and also creates opportunities for regulation. We also pose key unanswered questions in the interest of guiding future work in this area.

Constrained in time: Hedgehog signaling and cilia dynamics in proliferating cells

The dynamics of cilia assembly and disassembly during the cell cycle

Primary cilia have a close relationship to the cell cycle and are often associated with the quiescent cell cycle state. In many cell types, a cilium is assembled from a membrane-docked mature 'mother' centriole upon cell cycle exit into G0 (Fig. 2A). Accordingly, serum starvation, which results in cell cycle exit, is often used to induce ciliation in cell culture. Cell cycle re-entry from G0 is often accompanied by cilium disassembly (Pugacheva et al., 2007; Tucker et al., 1979), and several studies suggest that disassembly is required for re-entry. For example, depletion of the centrosome-associated proteins Tctex1 (Li et al., 2011; Saito et al., 2017), Cpap/Cenpj (Ding et al., 2019; Gabriel et al., 2016) or Nde1 (Kim et al.,

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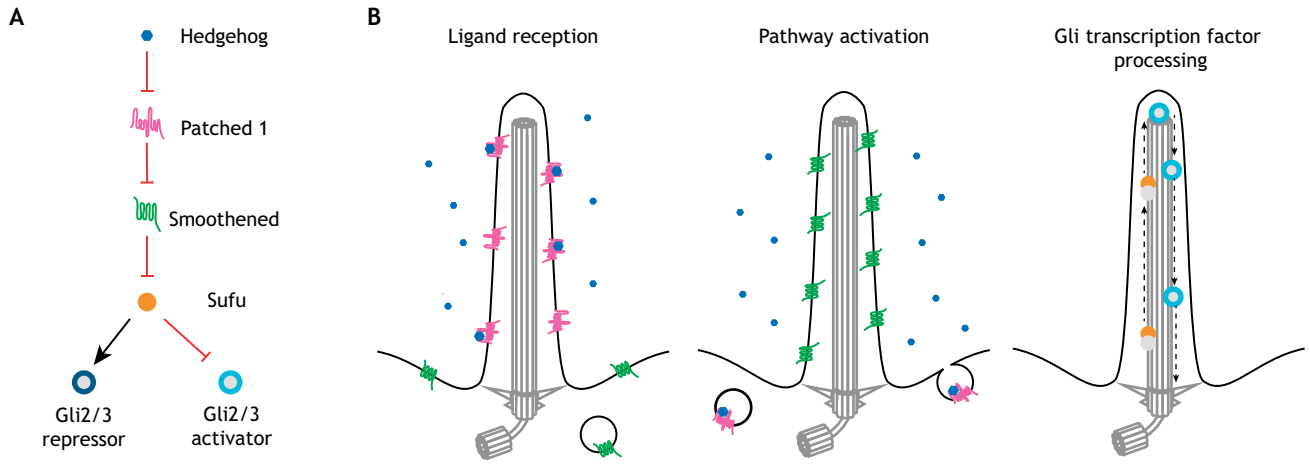

Fig. 1. Vertebrate Hh signaling requires the primary cilium for multiple aspects of signal transduction. (A) Schematic depicting the Hh signaling pathway. (B) Three parts of Hh signal transduction are thought to occur in cilia: ligand reception, pathway activation (via patched 1 and smoothened) and Gli transcription factor processing.

2011), among others, causes cilia disassembly defects and a delay in S-phase entry. Excision of the ciliary tip, an event likely associated with disassembly, has also been shown to be required for G1 entry from quiescence (Phua et al., 2017). Timely cilium disassembly is particularly relevant in the developing brain, where delayed cell cycle entry impairs neural progenitor production and is a cause of microcephaly (Ding et al., 2019; Doobin et al., 2016; Gabriel et al., 2016; Li et al., 2011; Zhang et al., 2019).

However, cilia are not found exclusively in quiescent cells. Proliferating cells in many developmental contexts and even some cancers possess cilia and require cilia and Hh signaling for their proliferation. Granule neuron precursors in the developing cerebellum are a well-studied example of a cell type that requires Hh pathway activity for proliferation, and loss of cilia in these cells leads to cell cycle arrest (Kenney and Rowitch, 2000; Spassky et al., 2008; Wechsler-Reya and Scott, 1999). Hh-dependent cancers, such as medulloblastoma and basal cell carcinoma, also require cilia for proliferation, unless mutations activate the pathway downstream of the essential cilium-mediated steps (Han et al., 2009; Wong et al., 2009). Thus, primary cilia can also promote rather than inhibit the cell cycle in contexts where they transduce proliferative signals.

In cycling cells, the cilium is only transiently present during the cell cycle. Cilia assemble in G1 and disassemble sometime before mitosis (Fig. 2B), although the exact timing is variable. Analysis of cells stimulated to enter the cell cycle by serum addition suggests that G1 and G2 are frequently points of cilium disassembly (Fig. 2C) (Pugacheva et al., 2007; Tucker et al., 1979). However, there is a need to study cilia disassembly in the context of normal cell cycles in culture and *in vivo*. Simultaneous imaging of cell cycle stage and cilia in mice found primary cilia on S/G2 cells in all tissues analyzed, suggesting that persistence of cilia past G1/S is common *in vivo* (Ford et al., 2018). Similarly, cilia in medulloblastoma cells persist through S phase (Ho et al., 2020), and cilia in dividing neural progenitors of the chick neural tube disassemble in late G2 (Spear and Erickson, 2012).

Although the timing of cilium disassembly is variable, the current understanding is that cilia must disassemble by mitosis. In general, mitotic cells lack cilia (Archer and Wheatley, 1971), and failure of cilium disassembly in chicken neural tube progenitors is correlated with absence of mitotic cells (Spear and Erickson, 2012). However, the requirement for cilium disassembly for successful completion of mitosis has not been rigorously examined, and cilium disassembly after entry into mitosis has been observed in PtK_1 cells (Rieder et al., 1979).

In summary, although the details of cilia dynamics during the cell cycle remain to be fully characterized, it is clear that the cilium is a transient organelle that is assembled and disassembled during each cell cycle. An important prediction then is that the potential of a cell to transduce Hh signaling might also be transient with respect to the cell cycle. If so, what are the implications of primary cilium transience on Hh signal transduction and cellular decisions informed by those signals?

The implications of cilia dynamics on Hedgehog signaling

The dynamic nature of cilia suggests that Hh signal transduction might not be constant throughout the cell cycle. Accordingly, it was recently shown that Hh signal transduction during the cell cycle is correlated with cilium presence in medulloblastoma cells (Ho et al., 2020). This cell cycle dependence is reminiscent of other signaling pathways with peaks of transduction at specific cell cycle phases (Davidson et al., 2009; Kim et al., 2019), but is unique in that the presence or absence of an organelle is the cause of the signaling changes. Although Hh signal transduction is generally correlated with cilium presence, there may be refractory periods following cilium assembly or prior to disassembly during which cilia are not competent for signaling due to structural changes or altered signaling component localization (Fig. 2D). Tagged Smo localizes to pre-ciliary membranes early in ciliogenesis (Lu et al., 2015; Wu et al., 2018), arguing against a delay in localizing signaling components to newly assembled cilia, although further characterization of signaling competence over the lifetime of a cilium is needed. It is also unknown whether signaling output is proportional to the duration of ciliation during the cell cycle (Fig. 2C). Just as cells regulate their ciliation state to modulate Hh signaling at developmental transitions (Chang et al., 2019; Das and Storey, 2014), perhaps cells can also fine-tune the duration of cilium presence during the cell cycle to maintain the optimal level of Hh signaling, potentially explaining the variability between cell types.

As well as showing variability between cell types, cilium assembly and disassembly are asynchronous within the same population (Fig. 2E). For example, the daughter cell inheriting the oldest centriole in a mitotic division typically assembles a cilium first (Anderson and Stearns, 2009; Paridaen et al., 2013; Piotrowska-Nitsche and Caspary, 2012). Moreover, live imaging

Development (2021) 148, dev195552. doi:10.1242/dev.195552

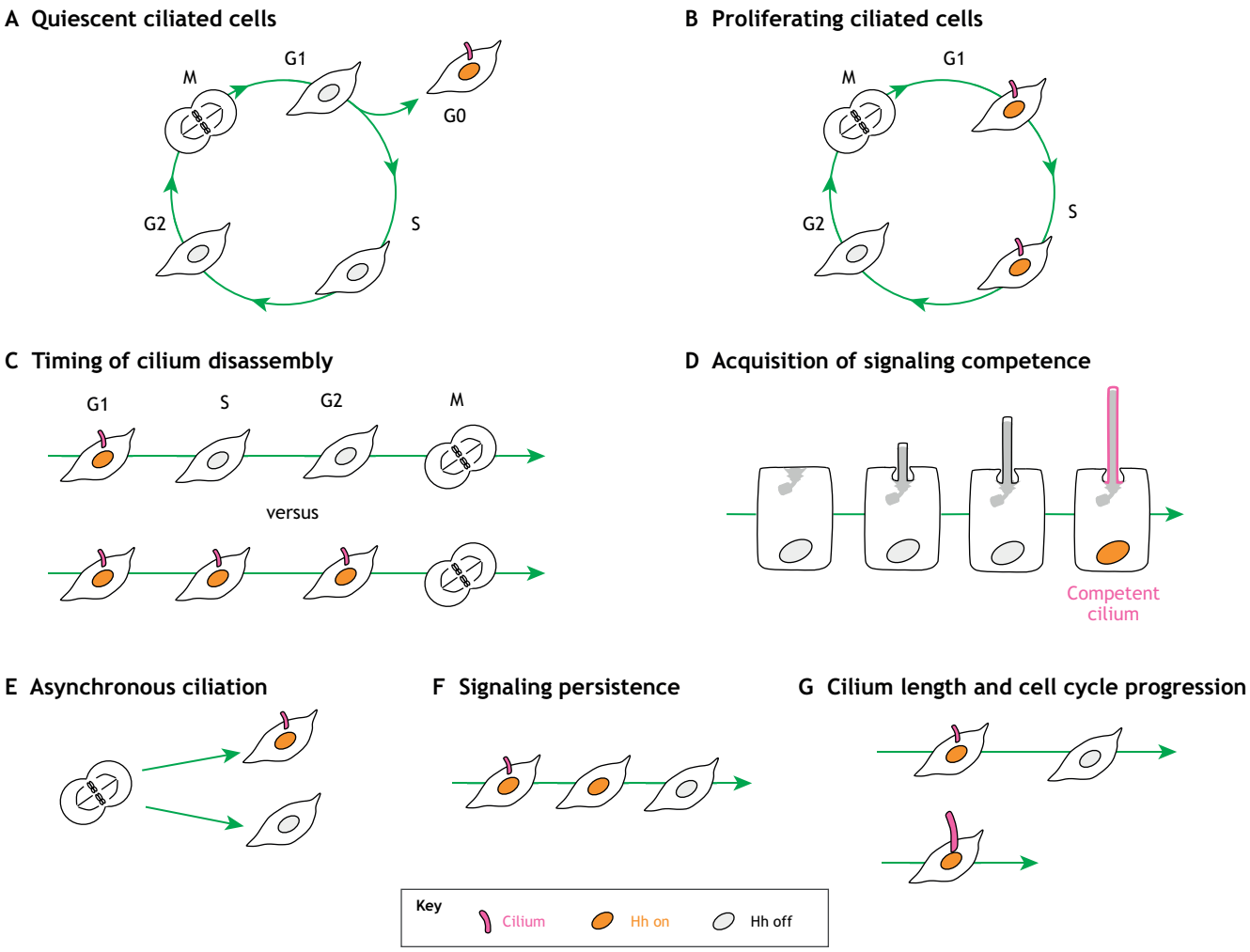

Fig. 2. Cilia dynamics in proliferating cells can impact Hh signal transduction. (A) Some cells only ciliate upon cell cycle exit, restricting Hh signal transduction (orange nucleus) to the quiescent (G0) cell state. (B) Other cells ciliate during the cell cycle, allowing Hh signal transduction when cilia are present. In these proliferating cells, several aspects of cilia dynamics influence Hh signal transduction. (C) The timing of cilia assembly and disassembly determines when cells can transduce Hh signaling; persistence of cilia until G2 allows signaling to occur over more of the cell cycle. (D) There may be a refractory period during cilia assembly in which newly assembled cilia are not yet competent for signal transduction. (E) Asynchronous ciliation between sister cells can lead to differences in Hh signaling. (F) There is evidence that some aspects of Hh pathway activity can persist after cilium disassembly. (G) Cilium length is important for regulating cell cycle entry as well as Hh signaling. This figure is drawn assuming a constant source of ligand.

of cilium disassembly shows it is a highly asynchronous and heterogeneous process; cilia disassemble by a variety of mechanisms, including rapid deciliation and gradual resorption (Mirvis et al., 2019). It has also been suggested that cilia may assemble and disassemble more than once within a single cell cycle (Spalluto et al., 2013; Tucker et al., 1979), although this phenomenon has not been observed directly by imaging.

Periods of the cell cycle in which a cell lacks a cilium likely cause gaps in Hh signaling, with the length of the gap depending on the timing of cilium assembly and disassembly in a given cycle. Although the initiation of ciliogenesis has been well characterized in cells that have exited the cell cycle, the factors controlling cilium assembly in cycling cells are not well understood. In non-cycling cells, serum withdrawal stimulates Cp110 removal from the distal end of the mother centriole (Čajánek and Nigg, 2014; Goetz et al., 2012; Xu et al., 2016), inhibition of the cilia disassembly factor Aurora A (Inaba et al., 2016; Inoko et al., 2012; Kasahara et al., 2014, 2018), autophagy-mediated degradation of the ciliogenesis inhibitor Ofd1 (Tang et al., 2013) and microtubule stabilization, which promotes apical centrosome migration (Pitaval et al., 2017). Recent work has linked serum starvation with the Rab11-Rab8 cascade that traffics vesicles to the centriole to initiate ciliogenesis (Walia et al., 2019). How these mechanisms apply in cycling cells that form cilia without imposition of serum starvation is unknown. Overall, a potential consequence of this variability in cilium formation is that cells in the same population may not experience equivalent amounts of Hh signaling. Are there developmental consequences of this variability or are there buffering mechanisms to mitigate them?

We propose that cells have mechanisms to ‘remember’ signaling between ciliated phases (Fig. 2F) as a means of dealing with the intrinsic variability of cilium presence. Evidence for such mechanisms comes from the neural tube, where developmental decisions are made based on the amount of Hh that cells are exposed to. Here, progenitors cells integrate Hh signal concentration and duration over time to control differential gene expression rather than measuring Hh signaling at a single point (Dessaud et al., 2007). These cells must then not only integrate signals over time but also

Development (2021) 148, dev195552. doi:10.1242/dev.195552

must do so as they are rapidly proliferating (Briscoe and Novitch, 2008; Kicheva et al., 2014). In support of this model, recent work has shown that Hh pathway activity from the previous cell cycle influences the decision to enter the cell cycle, revealing that cells can retain a memory of signaling between cell cycles (Ho et al., 2020). One aspect of this memory is persistence of cyclin D1 over times that span the ciliated signaling windows (Ho et al., 2020), although additional mechanisms likely exist. For example, it is possible that some part of the centrosome/cilium complex that persists between ciliated periods also contributes to memory of signaling. The ciliary remnant is a candidate for such a structure: it is a vesicle derived from the disassembled cilium that remains attached to the centriole, and it provides a memory of the ciliated state into the next cell cycle (Paridaen et al., 2013). In fact, Smo is present in the remnant following pathway stimulation, suggesting that Hh signaling components can be inherited in this way (Viol et al., 2020).

Cilium structure in the context of the cell cycle

Aspects of cilium structure can also influence signaling. The caliber of the primary cilium is thought to be similar in most cases, but cilium length can be highly variable between cell types. Importantly, changes in cilium length for a given cell type have consequences for both cell cycle progression and Hh signaling (Fig. 2G). Depletion of Nde1 or Cpap/Cenpj results in elongated cilia and a delay of cell cycle entry (Ding et al., 2019; Doobin et al., 2016; Gabriel et al., 2016; Kim et al., 2011). Mutations that affect cilium length are also often associated with defects in Hh signaling (Caspary et al., 2007; Huangfu and Anderson, 2005). Although aberrant cilium length is not always accompanied by altered Hh signaling (Bangs et al., 2015; Bonnafe et al., 2004; Gigante et al., 2020), the association with structural alterations is consistent with the observation that the correct concentration of signaling components in cilia is crucial for their signaling function (Mahjoub and Stearns, 2012). If cilium length is a tunable variable for a cell, is the optimum for Hh signal transduction the same as that for other functions associated with proliferation, or must Hh-responsive cycling cells make trade-offs that growth-arrested cells need not?

Another unexplored question is whether short-lived primary cilia on cycling cells (i.e. those present for only part of one cell cycle) are structurally different from long-lived cilia on differentiated cells, such as neurons or kidney epithelial cells. Is the structure of a cilium that must disassemble different from the structure of one that need not, and how might any differences affect Hh signaling? One hint comes from work on modifications to tubulin in the axoneme. In MDCK cells, prolonged starvation-induced cell cycle arrest causes the accumulation of tubulin glycylation modifications, which are associated with increased axoneme stability (Gadadhar et al., 2017). Although tubulin glycylation has not been studied in the context of Hh signaling, it is relevant that another tubulin modification, polyglutamylation, does influence Hh signaling. Reduced polyglutamylation impairs Gli3 transit to the cilium tip and consequently reduces Gli1 expression (He et al., 2018; Hong et al., 2018). Thus, it will be interesting to directly investigate how cilium age affects signaling capacity through structural changes. New advances in electron microscopy provide an exciting avenue to better understand variations in cilium structure between cell types and conditions (Kiesel et al., 2020; Sun et al., 2019).

We conclude that, unlike cells that are quiescent and have a long-lived primary cilium, cells in a proliferating tissue have dynamic primary cilia that are a source of variability in signal transduction capacity, both in a given cell through time and among cells in the same tissue. Although this variability is still largely uncharacterized, recent results have shown that considering Hh signaling in light of cilia dynamics can lead to insights into how the Hh pathway functions in developmental contexts. An exciting suggestion of these findings is that cells might control whether and when to elaborate a cilium, and to fine-tune the structure of that cilium to add additional layers of regulation onto Hh signal reception.

Constrained in space: Hedgehog signaling in ciliated epithelia

The location and positioning of cilia

The primary cilium is a small compartment compared with the size of a typical animal cell. Averaging about 5 µm in length, the primary cilium has an estimated volume that is about 5000 times smaller than the rest of the cell and a surface area about 500 times smaller (Nachury, 2014). Although this small size is thought to improve sensitivity by allowing a high local concentration of signaling components, a consequence is that cilium-based receptors are concentrated in only a small region of the cell membrane. If a cell is surrounded by uniform ligand concentration, as occurs in most cell culture experiments, the location of the solitary signaling organelle likely is of no consequence. But how does cilium position affect signaling in the complex milieu of the developing tissues of the embryo?

The location of the cilium on a cell is determined by the docking of the mature centriole to the plasma membrane. In epithelial cells, the initiation of ciliogenesis is marked by accumulation of ciliary vesicles at the end of this centriole as well as migration of the centriole toward the apical surface. Centriole migration to the apical surface requires actomyosin contractility and microtubule network remodeling (Hong et al., 2015; Pitaval et al., 2017). Actin is also cleared from the site of apical docking (Francis et al., 2011; Jewett et al., 2021), and a recent study (Insinna et al., 2019) identified a membrane channel connecting the ciliary vesicle to the plasma membrane during ciliogenesis that may guide the centriole to the apical membrane.

In epithelial cells, the cilium is almost exclusively an apical organelle, with rare exceptions, including some neuronal progenitors, exhibiting basolateral cilia (Lepanto et al., 2016; Wilsch-Brauninger et al., 2012). The apical location of cilia would seem to impose a spatial constraint on Hh signaling. Each cell in an epithelium has membrane contact with two compartments, apical and basal, separated by restrictive tight junctions and adherens junctions. Hh ligand may be present in either or both of these compartments, depending on the relative position of Hh-sending cells. However, the apical cilia project only into the apical compartment. So, how does the restricted location of the cilium affect the response of an epithelium to basal versus apical sources of Hh? To address this question, we examine current understanding of the spatial implications of Hh secretion, transport and reception, as well as examples of epithelial Hh signaling that provide insight into how signaling operates *in vivo*.

Ligand source and its relationship to cilium location in epithelia

In some epithelial signaling contexts, the spatial relationship between Hh ligand source and primary cilia is straightforward, with Hh in the same compartment as the primary cilia of the receiving cells. For example, Shh within the embryonic cerebrospinal fluid in mice has direct access to the apical primary cilia of radial glia, which project into the ventricle (Huang et al., 2010) (Fig. 3A). This choroid plexus-derived Shh is required for radial glia cell proliferation in the embryonic cerebellum prior to

Development (2021) 148, dev195552. doi:10.1242/dev.195552

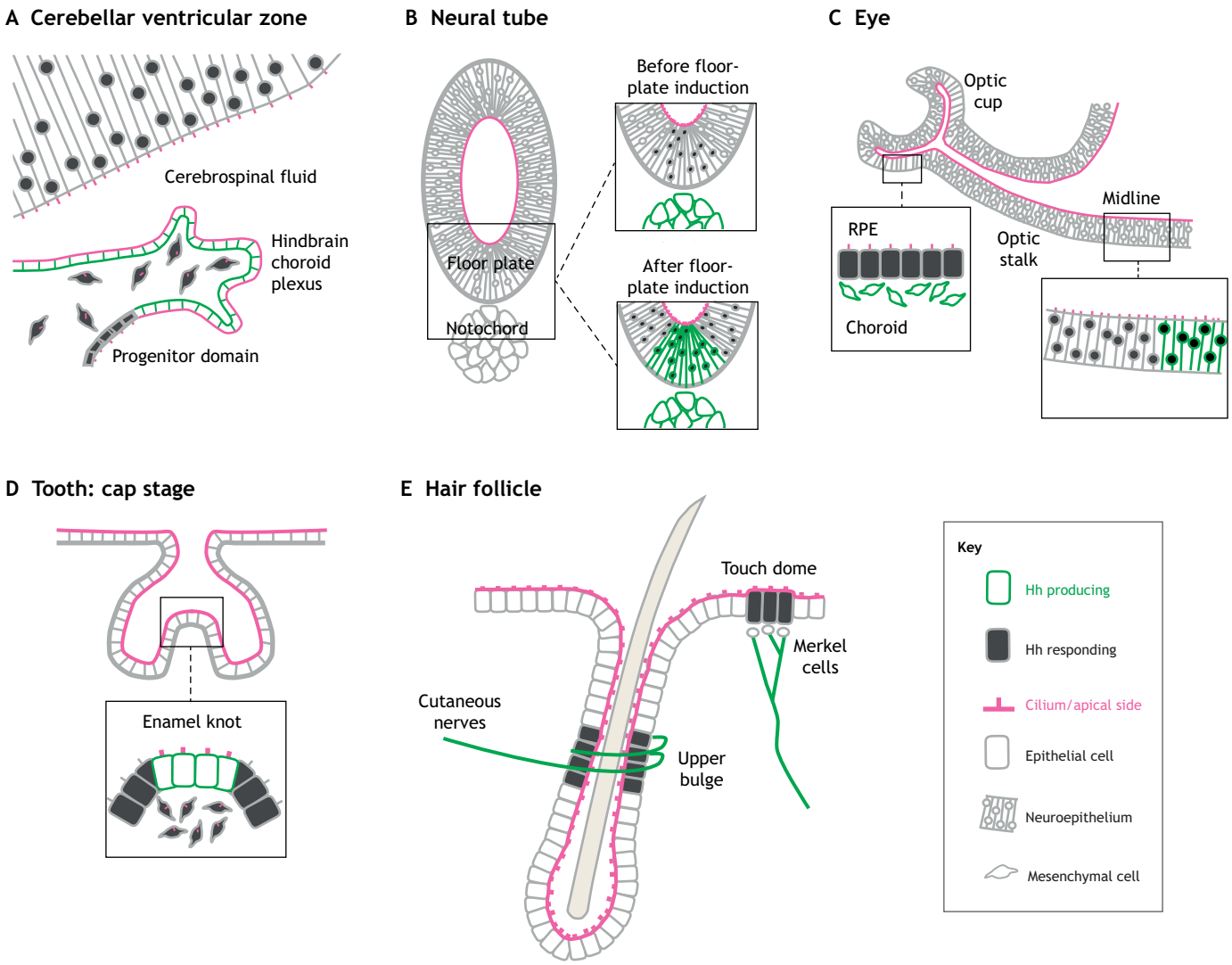

Fig. 3. The relationship between ligand source and cilia in Hh-responsive epithelia *in vivo*. Cells secreting Hh ligand are outlined in green and cells responding to that Hh source are filled with gray. Magenta highlights the apical side and cilia. (A) In the developing mouse brain, Shh from the hindbrain choroid plexus epithelium signals to the nearby cerebellum via the apical cerebrospinal fluid, to the adjacent progenitor domain and to the underlying mesenchyme. (B) During neural tube development, Shh from the notochord signals from the basal side to the overlying ventral neural tube, inducing the floor plate. The floor plate then begins production of Shh, which signals to the rest of the neural tube. (C) During eye development, Shh from the midline signals to the adjacent neuroepithelium, inducing the optic stalk. In addition, Ihh from the choroid signals to the maturing retinal pigment epithelium (RPE) from the basal side of the RPE. (D) At the cap stage of tooth development, Shh from the enamel knot signals to both the adjacent epithelium and the underlying mesenchyme. (E) In the hair follicle, cutaneous nerves signal to cells of the upper bulge. Within the skin, nerves innervating Merkel cells provide a source of Shh for the developing touch dome.

Shh production by the cerebellum itself (Huang et al., 2009, 2010). Shh and Ihh have also been detected in the apical fluid of the mouse epididymis, where they can access primary cilia of the epididymal epithelium (Girardet et al., 2020). Importantly, the apical presence of Hh ligand does not guarantee its access to primary cilia, as primary cilia only constitute a small proportion of the apical membrane. Thus, it is also relevant that there are many regulators of ligand transport and ligand-membrane interactions, including glypicans and co-receptors, that may influence how the Hh ligand interacts with the cilium (Capurro et al., 2008; Wierbowski et al., 2020).

In other tissues, the source of Hh is basal to the receiving cells, and the ligand is therefore separated from the primary cilium by the epithelial barrier. The classic example of this scenario is in the neural tube (Fig. 3B). Floor plate induction occurs when notochord-derived Shh signals to the most ventral neural tube cells. This notochord-derived signal is necessary and sufficient for floor plate induction (Matise et al., 1998; Patten and Placzek, 2002; Roelink et al., 1994; Tanabe et al., 1995). The apical cilia of the presumptive floor plate cells project into the lumen of the neural tube, but the notochord is on the basal side of the epithelium (Fig. 3B). Similarly, prior to maturation, cells in the retinal pigment epithelium (RPE) of the eye have apical cilia and respond to Ihh secreted by the developing choroid on the basal side of the epithelium (Dakubo et al., 2008; Lehmann et al., 2020; Portal et al., 2019) (Fig. 3C). Thus, there are well-defined examples of both apical and basal sources of Hh ligand involved in developmental signaling.

The spatial relationship between ligand source and primary cilia is not always so clear. For example, there are many examples of epithelial signaling centers that signal to other cells within the same

Development (2021) 148, dev195552. doi:10.1242/dev.195552

epithelium. This process occurs in the neural tube, where floor-plate cells, after being induced by the notochord, secrete their own Shh (Dessaud et al., 2008) (Fig. 3B). This floor-plate-derived Shh is required to maintain the neural progenitor domains in the neural tube established by notochord-derived Shh (Yu et al., 2013). Similarly, midline floor-plate cells signal to optic stalk cells during early eye morphogenesis (Chiang et al., 1996; Ekker et al., 1995; Macdonald et al., 1995) (Fig. 3C). Moreover, in tooth development, Shh is expressed by cells in the epithelial bud tip that will become the enamel knot, and signals to cells of the surrounding epithelium and underlying mesenchyme (Hardcastle et al., 1998; Wang et al., 2005) (Fig. 3D). In these cases, cilia are apical, but whether the ligand is apical or basal is unclear and requires further understanding of its secretion and transport.

Evidence from *Drosophila*, which does not require cilia for Hh signaling, shows that there are both apical and basal routes of Hh release in epithelia. Newly synthesized Hh ligand is initially presented apically, and then endocytosed and trafficked for apical or basal secretion (Ayers et al., 2010; Callejo et al., 2011; D'Angelo et al., 2015). Secretion primarily occurs in the form of exosomes, although there is also evidence for soluble and lipoprotein-associated ligand (Gradilla et al., 2014; Panáková et al., 2005; Zeng et al., 2001). Basally secreted Hh is transported along cytonemes: long actin-based filopodial extensions present on the basal side of *Drosophila* epithelial cells that are required for signal transport and reception (Bischoff et al., 2013; Chen et al., 2017; González-Méndez et al., 2017). A key player in Hh secretion is Dispatched, a multipass transmembrane protein closely related to the Hh receptor Patched. Dispatched is required for the apical endocytosis of Hh as well as its basal secretion (Callejo et al., 2011; D'Angelo et al., 2015). Its mammalian homolog dispatched 1 has a conserved function in the basal secretion of Shh (Etheridge et al., 2010).

Comparatively less is known about the polarity of Hh secretion in vertebrates. One study showed that Shh secretion from MDCK cells is primarily basal and dispatched 1 dependent, although some apical Shh is present (Etheridge et al., 2010). Basal secretion is consistent with the observation that Hh-producing epithelia often signal to underlying ciliated mesenchymal tissues, as in the mammalian prostate and bladder (Peng and Joyner, 2015; Roberts et al., 2017), choroid plexus (Fig. 3A) (Huang et al., 2009), and tooth (Fig. 3D) (Hardcastle et al., 1998). Cytonemes also have a role in vertebrate Hh signaling (Hall et al., 2021; Sanders et al., 2013), and have been identified on the basal side of the developing optic stalk epithelium (Cardozo et al., 2014). Interestingly, in the neural tube, Shh accumulation has been observed both on the basal side of the neuroepithelium (Gritli-Linde et al., 2001) and in a ventral-dorsal gradient in the apical lumen (Chamberlain et al., 2008), although the relative contributions from the floor plate are unclear because notochord-derived Shh is found in both locations. Thus, in the case of epithelial cells signaling to cells within the same epithelium, there is strong evidence for a basal source of ligand, although apical ligand may also be present.

Shh can also be produced by nerve cells, which innervate many epithelial tissues and therefore provide an external source of ligand. However, the spatial relationship between Shh source and cilia in these contexts remains largely uncharacterized. In the hair follicle, Shh-producing sensory neurons wrap around the upper bulge region of the follicle, activating Hh signaling in those epithelial cells (Brownell et al., 2011; Lehman et al., 2009) (Fig. 3E). In development and maintenance of the touch dome, Shh-producing nerves innervate specialized epithelial sensory cells termed Merkel cells, which are located basally to Hh-responsive, apically ciliated basal keratinocytes (Elofsson et al., 1984; Ezratty et al., 2011; Peterson et al., 2015; Xiao et al., 2015, 2016) (Fig. 3E). In the adult brain, Hh-producing neurons project processes to the ventral subventricular zone, where Shh promotes self-renewal of apically ciliated neuronal progenitors (Garcia et al., 2010; Ihrie et al., 2011; Petrova and Joyner, 2014; Tong et al., 2014). It remains unexplored how and where Hh ligand is released from these neurons to activate signaling.

These complex spatial relationships raise the issue of whether Hh ligand is able to move between apical and basal compartments. Evidence in favor of this comes from the observation that Shh is localized within the neural tube lumen, even in the absence of Shh production by the floor plate, suggesting it is derived from the notochord (Chamberlain et al., 2008; Christ et al., 2016; Himmelstein et al., 2017). Transcytosis has been proposed as a potential mechanism for this movement, supported by putative visualization of notochord-derived Shh within the floor plate (Chamberlain et al., 2008). Another possible explanation for these results is that Shh moves between – rather than through – the floor-plate cells. Indeed, at the time of floor-plate induction, junctions between neural tube cells loosen, changing the restrictive nature of this barrier (Aaku-Saraste et al., 1996). Shh might therefore move between cells by diffusion, although recent work in cell culture (Li et al., 2018) and the adrenal gland (Mateska et al., 2020) suggests that membrane contact with ciliated receiving cells, perhaps by the extension of thin cytonemes through the space between cells (Sanders et al., 2013), is necessary for Shh transport.

Overall, these examples demonstrate that the source of Hh ligand in Hh-responsive epithelia may be apical or basal, or both. Although further work is needed to fully define the sources and transport route taken for the Hh source for each tissue, the diversity of signaling configurations described here suggests that epithelia are able to respond to both basal and apical sources of Shh; whether that is directly or through transport of the ligand, however, remains to be determined.

Hedgehog reception and its relationship to the primary cilium

The predominant model for Hh signaling in mammalian cells is that Hh ligand must bind to its receptor Ptch1 in the ciliary membrane to activate signaling. This model is based on two major lines of evidence. First, Ptch1 localizes to and concentrates in the ciliary membrane when the pathway is in the off state; when ligand is added, Shh localizes to cilia and causes Ptch1 exit from cilia (Rohatgi et al., 2007). Second, localization to the cilium compartment is required for Ptch1 and Smo to function in signaling. Removing the C-terminal tail of Ptch1 disrupts cilia localization while maintaining ligand binding (Kim et al., 2015). This non-ciliary Ptch1 cannot inactivate Smo, as evidenced by its failure to rescue pathway activation in *Ptch1*$^{-/-}$ mouse embryonic fibroblasts. Similarly, Smo that cannot localize to the cilium does not activate the pathway (Corbit et al., 2005). Biochemical experiments have also provided insights into the mechanisms of Smo regulation by Ptch1 in cilia. For example, recent work has shown that cholesterol is an endogenous ligand for Smo and provides evidence that Ptch1 regulates Smo by modulating its access to cholesterol (Byrne et al., 2016; Huang et al., 2016; Luchetti et al., 2016; Petrov et al., 2021). Analysis of the lipid composition of cilia has revealed that cilia exhibit lower cholesterol accessibility compared with the plasma membrane, which may be important for regulating Smo activity (Kinnebrew et al., 2019). Together, these experiments suggest that cilia concentrate signaling components in an environment optimized for signal transduction.

Development (2021) 148, dev195552. doi:10.1242/dev.195552

The model of cilia as a specialized compartment for Hh reception projecting into a signal-rich environment is consistent with other modes of ciliary signaling. Motile cilia in the trachea contain bitter taste receptors that are thought to sense noxious chemicals in the airway and increase ciliary beating frequency (Shah et al., 2009). Olfactory GPCRs concentrate in olfactory cilia that project into the odorant-rich environment of the olfactory epithelium (Mykytyn and Askwith, 2017). It is interesting to note that, although most differentiated neurons have cilia localized to their cell bodies, olfactory and other sensory cilia are located at the tip of dendrites (Jenkins et al., 2009). This location of the olfactory cilium thus appears to be specialized specifically for its function. Concentration of Ptch1 in cilia may have a similar consequence for Hh signaling.

Other work, however, brings into question the requirement for Hh ligand binding to Ptch1 at cilia. Functional experiments have suggested that basal Shh is actually the relevant source of Shh during neural tube differentiation. Addition of the Shh-blocking antibody (5E1) to the apical lumen has no effect on Hh response in this context (Etheridge et al., 2010), and notochord grafting inside the apical lumen also has no effect on Hh target gene expression (Kahane and Kalcheim, 2020). Consistent with this model, there is evidence that, in vertebrates, as in *Drosophila* (González-Méndez et al., 2017), Hh receptors localize to the basal sides of cells. For example, the Hh co-receptor Cdon, which can negatively regulate the pathway by acting as a sink for ligand, is present on the basal side of the developing optic vesicle epithelium (Cardozo et al., 2014). Basal Ptch1 has also been described as a mediator of long-range Shh transport in embryonic stem cell cultures, although basal localization of Ptch1 has not been directly visualized (Etheridge et al., 2010).

There is also evidence that the interaction between Ptch1 and Smo can occur outside cilia. Reconstitution experiments have shown that Ptch1 can inhibit Smo activity outside of the specialized ciliary compartment (Myers et al., 2017). Examples of non-canonical, transcription-independent Shh signaling also provide evidence of these signaling molecules functioning outside of cilia. For example, Shh can regulate axon guidance by binding to Ptch1 and the co-receptor Boc at the growth cone, activating Smo to promote local cytoskeletal rearrangement and axon turning (Charron et al., 2003; Ferent et al., 2019a; Okada et al., 2006; Yam et al., 2009). The binding of Shh to Ptch1 occurs at a distance from the cell body where the cilium is located, and it has been shown that axon guidance in mouse commissural axons does not require cilia-localized Arl13b (Ferent et al., 2019b). Similarly, there is evidence that Shh-mediated chemotaxis in fibroblasts occurs independently of cilia (Bijlsma et al., 2012). Other work, however, shows that Arl13b in cilia is necessary for axon guidance in the chicken neural tube and chemotaxis in fibroblasts (Mariani et al., 2016; Toro-Tapia and Das, 2020), and so the issue of whether Ptch1 and Smo function outside of cilia remains unresolved.

Given the existing data, it is clear that the primary cilium is required to process and transduce Hh signals in mammalian cells, but we are not yet able to conclude whether it is also a necessary site of signal reception for Hh. Addressing this fundamental question of cilia biology will require directly testing whether Hh ligand must have access to the primary cilium to exert its signaling effect.

With our current understanding, we can propose a range of explanations to reconcile the apparent constraint of an apical antenna with the clear evidence for a basal source of ligand. One model is that Hh ligands undergo transcytosis to reach the apical cilium for reception. Hh ligands could be transcytosed alone or bound to a receptor (Fig. 4A). Although there is only limited direct evidence for transcytosis in the floor plate (Chamberlain et al., 2008), it is relevant to note that apico-basal trafficking of both Ptch and Hh does occur in *Drosophila*, which do not require cilia for Hh signaling. These apico-basal trafficking pathways might therefore be modified for signal reception in vertebrates (Callejo et al., 2011; González-Méndez et al., 2020). A variation of this model is that initial basal reception of ligand is a necessary step for signal transduction, perhaps mediating biochemical changes necessary for reception at cilia (Fig. 4B). This model is reminiscent of the finding that initial apical presentation of Hh ligand is necessary for proper secretion in *Drosophila* (D'Angelo et al., 2015). A second model is that the binding of Hh ligand to basal Ptch1 is sufficient to activate the pathway (Fig. 4C). This would require communication between the basal domain and the cilium, for which no mechanism currently exists. A third model is that, in cases where cells in an epithelium respond to a basal source of Hh ligand, the ligand can gain access to the apical compartment by moving between cells by diffusion (Fig. 4D) or even by cytoneme transport (Fig. 4E). Importantly, all of these models invoke the potential for developmental regulation of epithelial responsiveness to basal Shh ligand by modulating receptor localization, transcytosis or cell-cell junctions. We also note that some combination of these models is also possible; e.g. reception may occur at both ciliary and non-ciliary Ptch1, potentially explaining the observations that do not support a singular model.

Conclusions and future perspectives

This Review has explored the constraints imposed on the Hh pathway by the primary cilium. At first glance, the apical position and transient presence of the primary cilium would seem to impose limits on the time and place of ligand reception; this would presumably be balanced by the benefits of restricting Hh signaling to this organelle. Here, we have raised the possibility that these restrictions might also provide the potential for fine-tuned regulation of signaling in space and time. For example, regulation of ciliary dynamics or ligand localization would make it possible for some cells in a given context to be sensitive to a ligand while others nearby are not. In this regard, it has been shown that cilia submerged within a deep ciliary pit have different Hh signaling properties than fully exposed cilia, suggesting that changing the exposure of the cilium to the extracellular environment might be a regulatory mechanism to change signaling potential (Mazo et al., 2016). It is also interesting to consider that basal secretion of Hh ligand from ciliated epithelial cells into a different compartment from the apical primary cilia may provide an opportunity to prevent autocrine signaling activation of the epithelial cells themselves.

It is interesting to consider the implications of these constraints for *Drosophila* Hh signaling, which does not require the primary cilium. It has been proposed that some benefits of restricting signaling to a small, specialized compartment, such as the sensitivity to long-range signaling and the insulation from regulatory cholesterol sensing, are not necessary in *Drosophila* (Bangs and Anderson, 2016; Kinnebrew et al., 2019). Having identified the constraints imposed by the vertebrate primary cilium, we can further speculate that the opportunities for fine-tuning signaling that result from regulation of these constraints may be a vertebrate-specific evolutionary development. This advance might have occurred coincident with the establishment of a broader role for Hh signaling in dorsal-ventral patterning during neural development (Ren et al., 2020).

Our discussions here also highlight the need to develop new approaches for studying the Hh pathway in culture beyond serum-starved fibroblasts. Such G0 cells model neither the cilium dynamics that occur in cycling cells nor the separation of ligand and

Development (2021) 148, dev195552. doi:10.1242/dev.195552

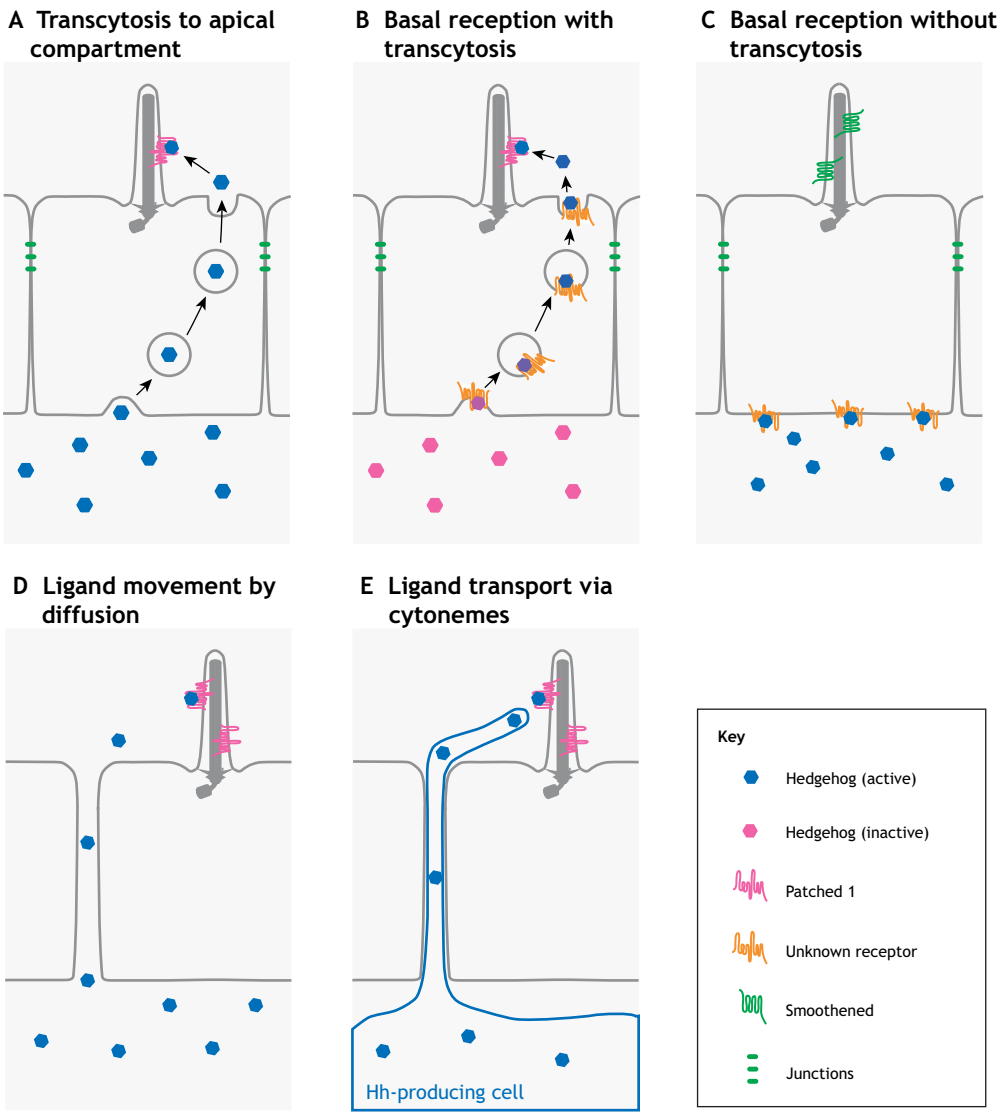

Fig. 4. Potential models for how epithelial cells respond to a basal source of Hh. (A) Active Hh ligand may undergo transcytosis to reach the apical compartment where it then binds to its receptor patched 1 (Ptch1) at the primary cilium. (B) Initial basal reception of inactive ligand may also be necessary prior to its reception at cilia. The basal receptor could be Ptch1 or another Hh-binding protein. (C) Another possibility is that Hh activates the pathway by binding to a receptor on the basal side. In this case, the Hh ligand itself would not reach the cilium but the pathway is still activated. (D,E) Alternatively, the epithelium may not restrict movement of Hh ligand between cells in tissues where signaling occurs from a basal source. In this model, Hh could move by diffusion (D) or transport (E) via filopodial extensions termed cytonemes. In these drawings, Hh is depicted as a soluble ligand but the models do not make that assumption.

cilium that often occurs in an epithelium. New medulloblastoma cell lines that maintain Hh pathway dependence in culture have been a useful development (Zhao et al., 2015, 2017), but more options are needed, especially to extend these findings to different cell types. Similarly, taking advantage of relevant Hh-responsive epithelial cultures, such as embryonic stem cell-derived 3D neuroepithelial cysts that model the neural tube (Meinhardt et al., 2014; Ranga et al., 2016), will be important.

Extension of genetic screening efforts to these more relevant systems will also be important for identifying unique regulators of Hh pathway-dependent proliferation. Although many screens have interrogated the molecular players in Hh signaling (Breslow et al., 2018; Pusapati et al., 2018; Rack et al., 2014; Wheway et al., 2015), these screens have been carried out in quiescent cells. As a result, any specific regulators of Hh pathway-dependent proliferation, including those in the pathway itself, those that regulate the assembly and disassembly of cilia in cycling cells, or those that mediate signaling memory through non-ciliated phases, would have been missed. Developing a screening approach for specifically identifying regulators of Hh pathway-dependent proliferation will be an important advance, and may be useful for identifying new drug targets.

New tools are also needed to fully address the mechanisms underlying these constraints. First, our understanding of cilia dynamics would benefit significantly from tools that allow simultaneous long-term imaging of the cell cycle and cilia dynamics. Progress in this area has been made using a combination of a Fucci cell cycle sensor and a tagged Arl13b cilia marker (Ford et al., 2018). This type of imaging will be very important to further define and compare cilia dynamics in a variety of cell types. Arl13b overexpression has known effects on cilia structure, so it will be important to validate this method with parallel approaches (Larkins et al., 2011). Similarly, imaging efforts should focus on visualizing the Hh ligand and the subcellular localization of its receptors. Thus far, it has been difficult to visualize Hh gradients in tissues or to fully characterize Hh binding locations on the cilium or cell surface.

It will also be necessary to develop new tools for controlling cilia dynamics. Although ciliation inhibitors exist, we have few tools to manipulate cilia once they are formed or to control in which phases of the cell cycle cells are ciliated. HDAC6 and Aurora A inhibitors that prevent cilia disassembly have been useful, but their independent effects on cell cycle progression limit their use (Pugacheva et al., 2007). Recent work shows that small molecule inhibition of the heterotrimeric ciliary kinesin 2 motor KIF3A/

KIF3B/KAP is a promising alternative approach for acutely inhibiting anterograde intraflagellar transport to the cilia tip, leading to cilia loss in fibroblasts within 8 h (Engelke et al., 2019). Although this hours-long time scale required for cilia loss is too long for cell cycle experiments, the developments in this area are exciting. Similarly, modulating cilium location on the cell would be important to address spatial constraints, and results using micropatterned substrates suggest some avenues for further work in making this possible (Pitaval et al., 2010).

This Review has focused on the Hh pathway because of its significance in development and its well-defined relationship to cilia. However, Hh signaling is not the only signaling pathway transduced in cilia. GPCR, PDGFα, IGF1, Wnt and Notch signaling pathways all have some requirement for cilia (Anvarian et al., 2019). The implications of cilium position and dynamics on Hh signaling thus likely also have similar but unexplored implications on these signaling pathways. For example, Notch receptors localize to cilia in the developing skin, and cilia are required for Notch activity in the epidermis (Ezratty et al., 2011). Given that Notch signaling requires direct contact between membrane-bound ligand and receptor, cilium placement may determine cell interactions. Exploring this and other implications of cilia biology on the many functions of these signaling pathways will be an important area of future research and will allow us to better understand this organelle and its extensive influence on development.

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