The mechanisms of Hedgehog signalling and its roles in development and disease

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Abstract | The cloning of the founding member of the Hedgehog (HH) family of secreted proteins two decades ago inaugurated a field that has diversified to encompass embryonic development, stem cell biology and tissue homeostasis. Interest in HH signalling increased when the pathway was implicated in several cancers and congenital syndromes. The mechanism of HH signalling is complex and remains incompletely understood. Nevertheless, studies have revealed novel biological insights into this system, including the function of HH lipidation in the secretion and transport of this ligand and details of the signal transduction pathway, which involves Patched 1, Smoothened and GLI proteins (Cubitus interruptus in *Drosophila melanogaster*), as well as, in vertebrates, primary cilia.

Morphogen
A protein that forms a gradient and directs tissue patterning.

Holoprosencephaly
Failure of the cephalic lobes to separate, which is associated with facial deformities.

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Hedgehog (Hh) was first identified by genetic screens in *Drosophila melanogaster*[1]. It earned its name from the appearance of embryos with null alleles of *hh*, which display a lawn of disorganized, hair-like bristles reminiscent of hedgehog spines. Its molecular identification occurred 12 years later, revealing it to be a secreted protein that directs pattern formation in adjacent cells[2]. Shortly thereafter, three mammalian counterparts, sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH), were found, and an evolutionarily conserved role for these molecules in body organization discovered[2–7]. SHH activity reproduces the actions of the zone of polarizing activity in the limb bud and of the notochord and floor plate in the neural tube[8,9], all of which are organizing centres that were described in classic grafting experiments decades ago. IHH regulates bone and cartilage development and is partly redundant with SHH, whereas DHH is essential for germ cell development in the testis and peripheral nerve sheath formation. The identification of these roles for HH proteins supported the textbook model of developmental biology, in which organizer tissues secrete a morphogen, HH in this case, that diffuses to trigger differential cellular responses according to its concentration. Almost simultaneously, interest in HH signalling came from another direction. Dysregulation of the HH pathway was found to be responsible for congenital syndromes, such as holoprosencephaly, and other developmental malformations[10]. Moreover, HH signalling was subsequently shown to regulate stem cell homeostasis in adult tissues, and persistent HH pathway activity seems to have pathological consequences in various cancers, including the skin cancer basal cell carcinoma and the brain tumour medulloblastoma[11].

Our purpose here is to provide readers with an update on HH signalling. We discuss how studying this pathway has provided fundamental insights into diverse areas of cell and developmental biology. We depict the unexpected complexity in the molecular mechanism of HH signal production, including how lipidation affects the secretion and extracellular spread of this ligand. We highlight specific aspects of the variation and conservation of HH signalling between species (for a review on the evolutionary origins of HH signalling, see REF. 12) and describe how the previously enigmatic primary cilia of vertebrate cells were found to function in HH signalling. We also review the multiple functions of HH and discuss how some of these diverse functions involve differential strength and duration of signalling, multiple feedback loops and non-canonical signalling.

HH maturation and secretion

Immature HH proteins undergo several post-translational modifications and cleavage events that modify their activity and regulate their spread from producing cells through tissues.

Dual lipidation of HH by cholesterol and palmitic acid. All HH proteins undergo signal sequence cleavage and enter the secretory pathway. Subsequently, HH molecules undergo autoproteolytic cleavage to generate an

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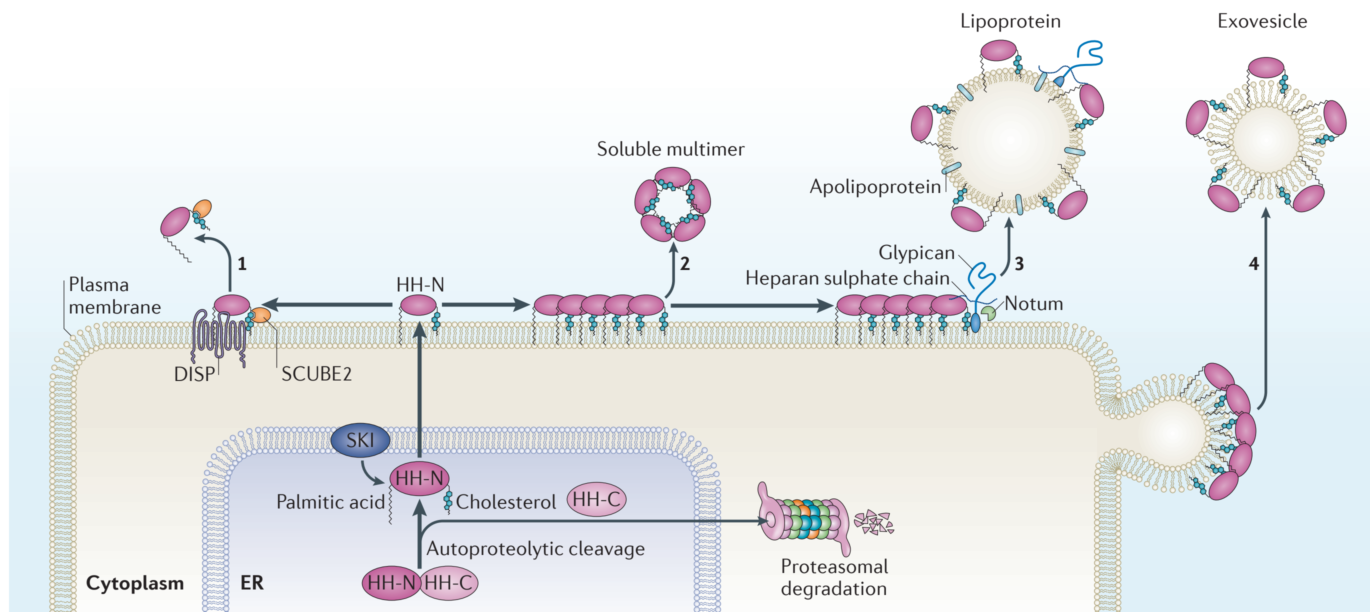

Figure 1 | **Hedgehog protein biogenesis and release.** Hedgehog (HH) proteins are synthesized as precursors of about 45 kDa. Autoproteolytic cleavage probably occurs in the endoplasmic reticulum (ER)[15]. The sterol recognition region of the HH carboxy-terminal peptide (HH-C) recruits cholesterol, which then acts as a nucleophile to attack an intramolecular thioester intermediate of HH. This results in the covalent attachment of cholesterol to the amino-terminal peptide (HH-N) and the dissociation of HH-C[14]. The acyltransferase skinny hedgehog (SKI) modifies HH-N by attaching a stable amide-linked palmitic acid group to the most N-terminal Cys residue, whereas HH-C translocates out of the ER and undergoes proteasomal degradation. Once at the outer surface of the plasma membrane, dually lipid-modified HH-N is associated with the lipid bilayer as a monomer until it is released by one of four key mechanisms. The cholesterol-modified HH-N monomer is released by the cooperative action of the transmembrane protein Dispatched (DISP) and the secreted SCUBE2 protein[29,40] (1). Both proteins bind directly to a different part of the cholesterol moiety of HH-N. Monomeric cholesterol-modified HH-N can also self-associate to form large soluble multimers that are released from the membrane[31,36] (2). HH-N oligomers can interact with the heparan sulphate chains of glypicans, which enables them to recruit lipophorin apolipoproteins and assemble into lipoprotein particles[49,50]. The glycosylphosphatidylinositol (GPI) anchor of glypican might be cleaved by the phospholipase C-like protein Notum, promoting the release of the HH-N-associated lipoprotein particles[214] (3). Alternatively, HH-N may be released at the surface of exovesicles[64] (4).

amino-terminal peptide that is linked to cholesterol at its carboxyl terminus (FIG. 1). All signalling activities are mediated by this N-terminal peptide, but the C-terminal region is necessary to catalyse the autoproteolytic cleavage event, a mechanism that is reminiscent of the protein splicing activity of inteins[13,14]. *In vitro* evidence suggests that the C-terminal fragment undergoes rapid degradation, a process that requires its translocation out of the endoplasmic reticulum (ER) to the proteasome[15]. HH cleavage is of importance, as mutations in human SHH that block the cleavage of the full-length protein cause the loss of SHH function, resulting in holoprosencephaly[16–18]. Notably, however, other studies have suggested that the C-terminal domain, independently of autoproteolytic cleavage, targets Hh to axons and growth cones in *D. melanogaster*[19]. Further study will be required to pin down the function of the C-terminal domain of HH.

HH is also modified by the attachment of a palmitic acid group, which greatly increases the activity of the protein in cell-based assays[20,21]. This acylation event is catalysed by skinny hedgehog (SKI), which is a member of the membrane-bound *O*-acyltransferase (MBOAT) protein family[14,22–26]. The subcellular compartment in which palmitoylation occurs has yet to be identified. However, engineered forms of vertebrate and *D. melanogaster* HH that lack cholesterol are palmitoylated inefficiently. This suggests that palmitoylation occurs after cholesterol modification[21], although *in vivo* the addition of cholesterol to HH is not a prerequisite for palmitoylation[27,28].

Inteins
Protein regions that are able to excise themselves and rejoin the remaining portions. This is the protein equivalent of an intron in a gene.

Spread of HH from producing cells. The distance HH travels to exert its effect seems to differ between tissues but can be up to 50 μm in the imaginal wing disc of *D. melanogaster* and up to 300 μm in the limb bud of vertebrates. The cholesterol modification of HH leads to its retention in the plasma membrane, restricting its free mobility[29,30], and both cholesterol and palmitic acid promote the association of HH with sterol-rich membrane microdomains[31,32]. This might be important for interactions with proteins involved in the release and spread of HH, such as the cytoplasmic membrane scaffolding protein Reggie 1, the *D. melanogaster* counterpart of vertebrate flotillin 2 (REF. 33). In *D. melanogaster*, nanoscale and larger visible oligomers of Hh have been described at the cell surface and depend on both cholesterol and a conserved residue that is involved in Hh homo-electrostatic interactions[34–36]. Disruption of these interactions adversely affects long-range activity, highlighting the importance of HH oligomerization for the spread of this molecule.

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Resistance-nodulation-division (RND) transporter family
A family of bacterial pumps that use a proton gradient to transport small lipophilic molecules across the membrane.

Glypicans
A family of heparan sulphate proteoglycans, members of which are anchored to cells via glycosylphosphatidylinositol (GPI).

Lipophorin
A scaffolding apolipoprotein that is highly enriched in *Drosophila melanogaster* haemolymph. Lipophorin is termed lipoprotein in vertebrates.

Dispatched (DISP), a multipass transmembrane protein from the resistance-nodulation-division (RND) transporter family[37–39], is required for the secretion of lipidated HH proteins. DISP binds directly to the cholesterol moiety of human SHH and acts in synergy with the vertebrate-specific SCUBE2, a secreted glycoprotein that binds to a different part of the cholesterol molecule, to promote the release of SHH from the cell surface[29,40] (FIG. 1). It is unclear in which cellular compartment DISP is required, but it is tempting to speculate that DISP transfers cholesterol-modified HH to SCUBE2 at the plasma membrane, so that the cholesterol anchor of HH is shielded from the aqueous environment. Independently, in *D. melanogaster,* Disp has been shown to be necessary for apicobasal trafficking of Hh; whether this shuttling promotes HH exposure to SCUBE2 in vertebrates is currently unclear[27]. Other studies have suggested that secreted proteases dissociate SHH from its two lipid moieties *in vitro*, resulting in SHH solubilization[41]. However, this mechanism has yet to be demonstrated *in vivo*.

Soluble cholesterol-modified HH ligands are found as monomers and as large multimers[31,35,36,42,43] (FIG. 1). HH proteins lacking cholesterol or palmitate cannot form multimers, and this results in defects in the long-range spread and signalling of these molecules[27,28,31,35,36,44,45]. How the multimers form remains an open question.

In *D. melanogaster*, glypicans stabilize and recruit Hh into visible clusters on Hh-producing cells[34,46–48]. Glypicans recruit circulating lipophorin to Hh-secreting cells and promote the association of Hh with lipoproteins through interactions between their glycosaminoglycan (GAG) chains[49,50]. Notably, removing circulating lipophorin reduces the range of Hh activity. The glypican glycosylphosphatidylinositol (GPI) anchor might be cleaved from the cell surface by a phospholipase C-like protein, allowing the release and spread of Hh in large soluble lipoprotein particles[46,49,51]. Another secreted protein from *D. melanogaster*, Shifted (Shf), which shares sequence similarity with the vertebrate WNT inhibitory factor (WIF), has also been shown to associate with both cholesterol-modified Hh and heparan sulphate proteoglycans (HSPGs). It remains unclear whether Shf is associated with the Hh-containing lipoprotein particles, but its loss decreases Hh stability and movement in *D. melanogaster*, but not in vertebrates[52,53].

Glypicans are also important for the spread of Hh through tissue. Cholesterol-modified Hh cannot cross a field of cells depleted of HSPGs in *D. melanogaster* imaginal discs, raising the possibility that Hh and its carriers are transferred by planar diffusion on glypicans of adjoining cells[54,55]. In vertebrates, the situation is complicated by the pleiotropic functions of glypicans (see below), but defects in the synthesis of the heparan sulphate chains of proteoglycans affect the distribution and activity of IHH during endochondral ossification[56], and HSPG sulphation modulates SHH spread in vertebrates as it does in *D. melanogaster*[57,58]. Thus, glypicans seem to have a conserved role in the spread of HH ligands.

Membrane proteins involved in HH reception also regulate the spread of HH proteins. For example, Patched 1 (PTC), one of the HH receptors (see below), and the vertebrate-specific HH-interacting protein 1 (HIP1) sequester HH to limit its diffusion[59–61]. Both proteins are induced by HH signalling and, thus, participate in ligand-dependent negative feedback control and have a substantial impact on the range of ligand availability. By contrast, proteins acting as HH co-receptors (see below) can also limit the range of HH signalling, but their expression is downregulated by HH. These feedback loops are probably necessary to refine the multiple thresholds of HH signalling within the target field.

Taken together, the current view of HH spread suggests that HH proteins are assembled into specialized vehicles to escape the plasma membrane of producing cells and spread through tissue. Nevertheless, mechanisms that involve the spread of HH on filopodia-like extensions of cells have also been proposed[62,63], and the various potential transportation mechanisms that HH might use, including transportation on extracellular vesicular particles (exovesicles), are outlined in BOX 1 and FIG. 1. These highlight the importance of future studies to determine whether similar or distinct mechanisms are used in different tissues and whether this reflects the distance that HH needs to travel or a specific tissue substrate over which HH needs to act[64]. Moreover, several isoforms and/or pools of HH might be produced from the same cells. Recently, endogenously produced Hh was purified from *D. melanogaster*, and sterol and non-sterol modified Hh molecules with complementary functions were identified[65]. Furthermore, a recent study suggested that the Hh gradient in the *D. melanogaster* wing imaginal disc is a composite of pools secreted by different routes: an apically secreted pool with long-range activity and a more basolateral secreted pool with short-range activity[46]. The identification of the different mechanisms and carriers controlling the formation and routing of HH will represent a major challenge in the next few years.

Box 1 | **Multiple carriers for Hedgehog transport**

In addition to the soluble multimers and lipoproteins, there is compelling evidence for the existence of other carriers of Hedgehog (HH), perhaps reflecting the diverse functions of this morphogen. For example, several studies have suggested that HH is transported in extracellular vesicular particles (exovesicles) (FIG. 1). Exosomes, which are exovesicles originating from endocytic multivesicular bodies, may carry HH-related peptides involved in cuticle formation in *Caenorhabditis elegans*[212]. Sonic hedgehog (SHH) has also been observed in exovesicles derived from apical microvilli budding at the surface of the mouse ventral node during embryonic development[213]. As other functional signalling molecules, such as Notch and Wingless (WG), have been detected on extracellular vesicles (reviewed in REF. 64), this type of transportation is an attractive hypothetical mechanism for the spread of HH that should be investigated further.

Finally, modes of transportation that do not depend on the release of HH from producing cells have been reported. In *Drosophila melanogaster*, Hh has been shown to decorate long basal filopodia[48], dubbed cytonemes, and it has been suggested that these cellular extensions deliver Hh at some distance from niche cells for the maintenance of germline stem cells[62]. These extensions are highly dynamic and might extend the signalling range of Hh. Recently, similar cytoneme-like structures carrying HH have also been reported in vertebrates[214]. Intracellular transport from the cell body to axons could also mediate the long-range transport of HH. In the *D. melanogaster* photoreceptor neurons and in planaria, HH is transported along the axons to induce postsynaptic neuron differentiation and to stimulate regeneration, respectively[19].

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Sterol-sensing domain
(SSD). A conserved portion of the resistance-nodulation-division (RND) domain. It was originally identified in proteins that have essential roles in the regulation of cholesterol homeostasis.

Oxysterols
Natural molecules derived from cholesterol oxidation.

The HH signalling pathway

Genetic analysis in *D. melanogaster* has identified the major components of the Hh pathway[66], which are conserved in diverse eumetazoa, including the non-bilaterian cnidaria. The first HH receptor identified, PTC, is a transmembrane protein that constitutively represses HH signalling[67,68] (FIG. 2). HH binding to PTC inhibits the repression of Smoothened (SMO), which is a member of the G protein-coupled receptor (GPCR) superfamily. This derepression results in the activation of the only known transcriptional mediators of the HH response, zinc-finger proteins of the GLI (also known as Cubitus interruptus (Ci) in *D. melanogaster*) family[10]. These are bifunctional transcription factors that can both activate or inhibit transcription. Regulation of the processing and nuclear translocation of GLI proteins has a key role in the HH signalling cascade (see below).

HH receptor complexes. In addition to PTC, several cell surface proteins that bind HH and promote signalling have been identified[69]. These co-receptors form separate multimolecular complexes with PTC and are required for high-affinity HH binding. They include the conserved and structurally related Interference Hedgehog (Ihog) and Brother of Ihog (Boi) in *D. melanogaster*, and their vertebrate orthologues CAM-related/downregulated by oncogenes (CDO) and brother of CDO (BOC), as well as growth arrest-specific 1 (GAS1), which is specific to vertebrates. Ihog, Boi, CDO and BOC are single-pass transmembrane proteins with immunoglobulin (Ig) and fibronectin type III (FNIII) repeats, whereas GAS1 is a GPI-linked protein with an extracellular domain homologous to glial cell-derived neurotrophic factor (GDNF) receptors.

HH binds directly to Ihog and CDO through different non-orthologous FNIII repeat domains[70]. Curiously, the crystal structures suggest that the mode of HH binding by FNIII domains is not the same in *D. melanogaster* and vertebrates, as the HH surfaces interacting with Ihog or CDO do not overlap and different cofactor dependencies have been identified: heparin is required for Hh–Ihog interactions and Ca^{2+} is required for SHH–CDO interactions[70]. GAS1, CDO and BOC also bind PTC to form a multimolecular receptor complex (Ihog–Boi–Ptc in *D. melanogaster*) that facilitates transduction. The binding of Ihog to Ptc is essential for the presentation of Ptc at the cell surface. Surprisingly, in various mouse tissues, only the absence of all three molecules, GAS1, BOC and CDO, results in the complete loss of HH activity[71,72]. This fact, along with biochemical findings, indicate that GAS1 binds PTC separately from the BOC–PTC and CDO–PTC dimers, suggesting that GAS1, BOC and CDO act collectively as co-receptors for SHH[71,72]. Nevertheless, despite their mutual contribution to the formation of a multimolecular complex, the GAS1, CDO and BOC co-receptors and PTC have opposing roles in pathway regulation — the co-receptors promote HH signalling and PTC inhibits it.

HH signalling is also regulated by glypicans, which enhance the stability of HH and promote its internalization with PTC[73]. Consistent with this, SHH proteins interact with the extracellular matrix component heparin and with heparan sulphate chains on proteoglycans through two distinct domains; the loss of these interactions reduces HH signalling potency[74–76]. Moreover, heparin is required for the dimerization of Ihog and its high-affinity interaction with *D. melanogaster* Hh[77]. Nevertheless, there is no clear model for how glypicans regulate HH, although it is plausible that specific glypicans enhance HH–PTC binding and signalling, whereas others compete with PTC for HH binding and inhibit signalling[73,78]. In some cases, the heparan sulphate chains are not necessary for the glypican core protein to fully restore HH signalling[78,79].

Other potential HH co-receptors have been identified, such as the low-density lipoprotein receptor-related protein 2 (LRP2; also known as megalin), which is required in the forebrain epithelium for SHH signalling and might be involved in PTC trafficking[80]. In conclusion, HH is multivalent and does not bind exclusively to a single receptor. The mechanism underlying the regulation of the multireceptor complex has yet to be elucidated and is a matter of great importance for future research.

Regulation of SMO by PTC. How PTC represses SMO activity remains unclear. Like DISP, PTC contains a sterol-sensing domain (SSD) and has structural similarity to members of the RND transporter family[81–83]. Mutations affecting the RND permease motif of PTC abrogate the PTC-mediated repression of SMO, leading to the suggestion that PTC controls the influx or the efflux of a ligand that controls SMO[84]. SMO contains a highly conserved extracellular N-terminal Cys-rich domain (CRD), which is required for its dimerization and function[85], but unlike other closely related GPCRs[86], no ligand-binding function has been assigned to it. By contrast, several naturally occurring and synthetic agonists and antagonists of vertebrate SMO have been shown to bind to its membrane-integrated heptahelical domain[87]. Many of these are structurally related to sterols, raising the possibility that an endogenous sterol-like molecule — an activating or repressing ligand — that is transported across the membrane by PTC regulates SMO. The most promising candidates for the activating ligand are oxysterols, which directly bind SMO and promote SHH signalling[88–90]. However, a sterol transporting function of PTC has yet to be demonstrated, and whether PTC transports an activating ligand (such as oxysterols) away from SMO, or an inhibitory ligand to SMO, remains to be clarified. Moreover, whether similar ligands also regulate *D. melanogaster* Smo is unclear.

A further clue to the mechanism of SMO regulation by PTC has come from studies in *D. melanogaster*. In flies, the inhibition of Ptc by Hh results in the accumulation of Smo at the plasma membrane as a consequence of an increase in its trafficking from internal vesicles and/or an increase in its stability[91–93]. Ptc has been shown to inhibit this process and recruit internalized lipophorin to Smo-enriched endosomes[94,95]. This has led to the suggestion that lipophorin may deliver sterol derivatives to Ptc and that these derivatives are responsible for negatively regulating Smo activity[94,95]. Consistent with this hypothesis, decreases in circulating lipophorin levels increase

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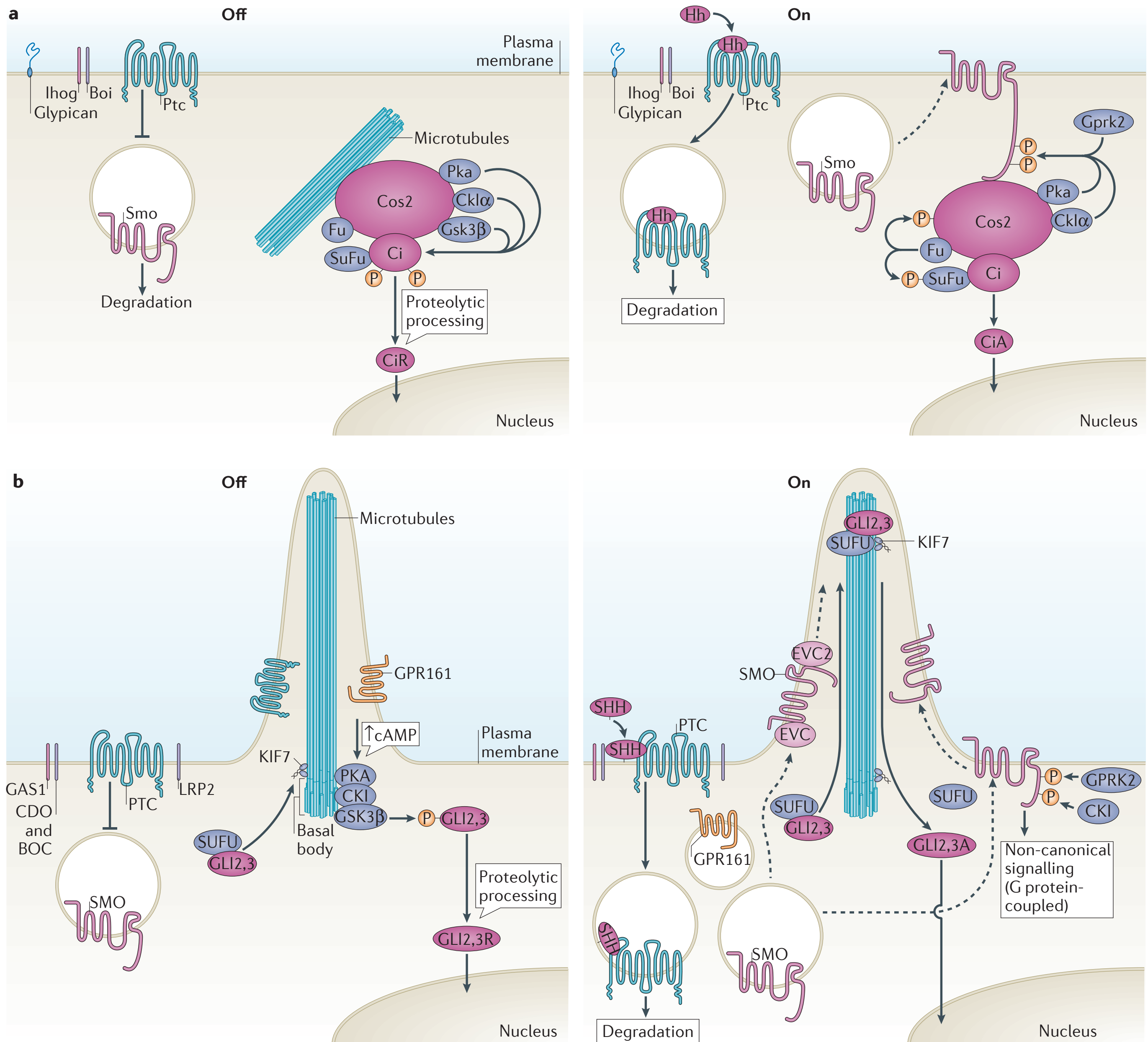

Figure 2 | **Reception of Hedgehog and initiation of signal transduction.** **a** | In *Drosophila melanogaster* , the transmembrane proteins Interference Hedgehog (Ihog) and Brother of Ihog (Boi) promote Hedgehog (Hh)–Patched 1 (Ptc) binding. Hh also interacts with glypicans. Ligand-free Ptc represses Smo by triggering its rapid degradation and/or its confinement to an intracellular compartment (left panel, 'off'). Furthermore, in the absence of Hh, the Hh signalling complex (HSC), which includes Costal 2 (Cos2), Fused (Fu), Suppressor of Fu (SuFu) and Cubitus interruptus (Ci), is associated with microtubules. This complex promotes, through the activity of protein kinase A (Pka), casein kinase Iα (CkIα) and glycogen synthase kinase 3β (Gsk3β) , the formation of the Ci repressor form (CiR). Binding of Hh to Ptc relieves Smo repression. Smo translocates to the membrane and is activated by phosphorylation on its carboxy terminal tail, by PKA, CkIα and G protein-coupled receptor (Gpcr) kinase 2 (Gprk2), which induces a conformational change[85]. This promotes its association with the HSC and the sequential activation of Fu and Cos2, which releases uncleaved Ci from the HSC to result in the activation of Ci (CiA)[46,118]. **b** | In vertebrates CAM-related/downregulated by oncogenes (CDO), brother of CDO (BOC), growth arrest-specific 1 (GAS1) and low-density lipoprotein receptor-related protein 2 (LRP2) promote HH–PTC binding[69]. PTC is enriched in and around the primary cilium[141], where it acts via a poorly characterized mechanism that might involve lipid transport to inhibit SMO activity (left panel, 'off'). In the absence of sonic hedgehog (SHH), at the base of cilia, the GLI proteins GLI2 and GLI3 are phosphorylated by PKA, CKI and GSK3β (PKA might be activated by an increase in cAMP generated by GPR161). This leads to their proteolytic cleavage to generate the repressor forms (GLI2R and GLI3R, respectively). In the presence of SHH ligand, PTC and GPR161 exit the cilia, and SMO is phosphorylated by GPRK2 and CKI and gated into the primary cilium in association with β-arrestin and the microtubule motor KIF3A[155,156] (right panel, 'on'; β-arrestin and KIF3A not shown). Within the cilium, SMO is enriched proximally in association with EVC (Ellis-van Creveld syndrome protein) and EVC2 (REFS 157,158). The activation of SMO results in an increased cilia dwell time for SUFU and GLI2 and GLI3, the dissociation of the GLI–SUFU complex within the cilia and the transport of full-length, activated GLI2 and GLI3 proteins from the cilia to the nucleus bypassing proteolytic processing. The movement of GLI2 and GLI3 through cilia is dependent in part on KIF7, the vertebrate Cos2 orthologue[140]. Activated SMO has also been proposed to initiate non-canonical HH signalling outside cilia in the form of GPCR signalling.

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Smo stability at the plasma membrane independently of Ptc in *D. melanogaster* wing imaginal disc. Thus, Ptc could be responsible for modifying the lipid composition of the endosomes through which Smo is trafficked, blocking its trafficking to the plasma membrane. Alongside these studies, increases in the level of the phospholipid phosphatidylinositol-4-phosphate (PtdIns(4)P) in *D. melanogaster* and mammalian fibroblasts have recently been shown to stabilize SMO proteins at the plasma membrane and increase HH signalling[96]. On the basis of genetic interactions it has been suggested that PTC maintains low levels of PtdIns(4)P in the cell cortex by downregulating the STT4 kinase activity responsible for converting PtdIns into PtdIns(4)P. As PtdIns(4)P is a precursor of signalling PtdIns, further studies are required to determine the link between PtdInsP, any lipid regulator of SMO and intracellular trafficking.

Activation of SMO also involves a conformational switch. In the absence of HH ligand, SMO is found as a dimer in which the cytoplasmic tails are in a closed (inactive) conformation. This conformation is maintained by electrostatic interactions in the C-terminal domain between positively charged Arg and negatively charged Asp clusters[85]. HH activation neutralizes the Arg cluster by triggering the sequential phosphorylation of a domain adjacent to it, promoting the conversion of SMO to an open conformation. This switch seems to be essential for the cell surface accumulation of SMO and signal transduction[85,97]. In *D. melanogaster*, Smo is phosphorylated successively by protein kinase A (Pka), casein kinase Iα (CkIα), CkII and Gpcr kinase 2 (Gprk2)[97–99]. Moreover, the various states of Smo phosphorylation are regulated by a set of protein phosphatases that act on these phosphorylated residues, including Pp1, Pp2a and Pp4 (REFS. 100,101). Genetic studies have shown that differences in the strength of Hh signalling are generated by the gradual phosphorylation of Smo, with a greater degree of phosphorylation corresponding to stronger signalling, probably as a result of a gradual change in the conformation of the cytoplasmic tail[99]. Strikingly, the cytoplasmic tail of vertebrate SMO has diverged substantially from that of *D. melanogaster* Smo, and lacks the cluster of Pka phosphorylation sites responsible for *D. melanogaster* Smo activation. Pharmacological inhibitors of PKA block SMO activation in vertebrate cells[102,103], but there is no evidence that PKA phosphorylates SMO directly, and genetic ablation of PKA does not seem to affect SMO[104]. Despite this divergence, however, mouse SMO is regulated by an analogous mechanism involving CKIα and GPRK2 phosphorylation[105]. It will be important to determine whether HH signalling regulates the activity or the distribution of these kinases and phosphatases.

HH signalling from SMO to Ci and GLI. Although SMO is a member of the GPCR family, whether SMO couples to G proteins remains a matter of debate. In *D. melanogaster*, *Xenopus laevis* and cultured fibroblasts, the transduction of SMO activation has been suggested to depend on the $G\alpha_i$ subunit[106], which inhibits adenylyl cyclase and, thus, the formation of cyclic AMP (cAMP) and the activation of PKA. However, these findings are at odds with previous studies in *D. melanogaster* showing that a cAMP-insensitive form of Pka does not interfere with normal Hh signalling and studies indicating that Smo is activated by Pka-dependent phosphorylation[102,103]; further investigation is needed to resolve this discrepancy.

Ultimately, the HH transduction pathway culminates in the regulation of Ci and GLI protein activity[10]. Three genes comprise this family in amniotes (GLI1, GLI2 and GLI3), whereas a single ancestral gene encoding Ci is present in amphioxus, ciona and *Drosophila* species[12]. All GLI and Ci proteins seem to have similar DNA-binding specificities, imparted by five tandem C2H2 zinc-fingers that comprise the DNA-binding domain. They all contain a C-terminal activation domain, whereas only Ci, GLI2 and GLI3 contain an N-terminal repressor domain (FIG. 3).

HH signalling results in a change in the balance between the activator and repressor forms of the Ci and GLI proteins by regulating their post-translational proteolytic processing. In the absence of HH signalling, the C-terminal domain of Ci undergoes sequential phosphorylation on multiple sites, first by PKA, which primes sites for additional phosphorylation at nearby residues by glycogen synthase kinase 3β (GSK3β) and different members of the CKI family[10,107]. This generates a binding site for the F-box-containing protein β-transducin repeat-containing protein (βTrCP; also known as Slimb in *D. melanogaster*), which recruits the S phase associated protein kinase 1 (SKP1)–cullin 1 (CUL1)–F-box protein (SCF) E3 ubiquitin ligase complex[108,109]. Ubiquitylation of specific residues then targets the modified protein to the proteasome, where the C-terminal transactivation domain is removed by partial degradation (FIG. 3a). The remaining protein, which comprises the N-terminal and DNA-binding domains, translocates to the nucleus, binds genomic target sites and represses transcription. Activation of Smo inhibits the phosphorylation and partial proteolytic processing of Ci, allowing full length Ci to act as a transcriptional activator.

The mechanism of GLI2 and GLI3 regulation in vertebrates seems broadly similar to Ci. Nevertheless, the roles of GLI2 and GLI3 have become more specialized. Mouse mutant studies indicate that GLI2 is chiefly responsible for the activator function in response to HH signalling, and GLI3 is responsible for the repressor activity[110,111]. Whether this reflects the greater efficiency of GLI3 processing to its repressor form compared with GLI2 processing[112] or the relative strength of the transcriptional activator and inhibitor domains of the two proteins, remains to be determined. GLI1 has diverged evolutionarily, lacking a transcriptional repressor domain and, in mammals, it seems to have only a minor role in amplifying the transcriptional response[113]. Different phosphorylation events have been found to either inhibit or potentiate GLI1 transcriptional activator function[114,115], but the details of these mechanisms remain to be resolved. In contrast to mammals, Gli1 is essential for normal Hh responses in zebrafish[116], suggesting some divergence of function within vertebrates. Moreover, amphioxus has a single *Gli* gene but, unlike *D. melanogaster ci*, this gene is alternatively spliced to produce gene products that act as either activators or inhibitors[117].

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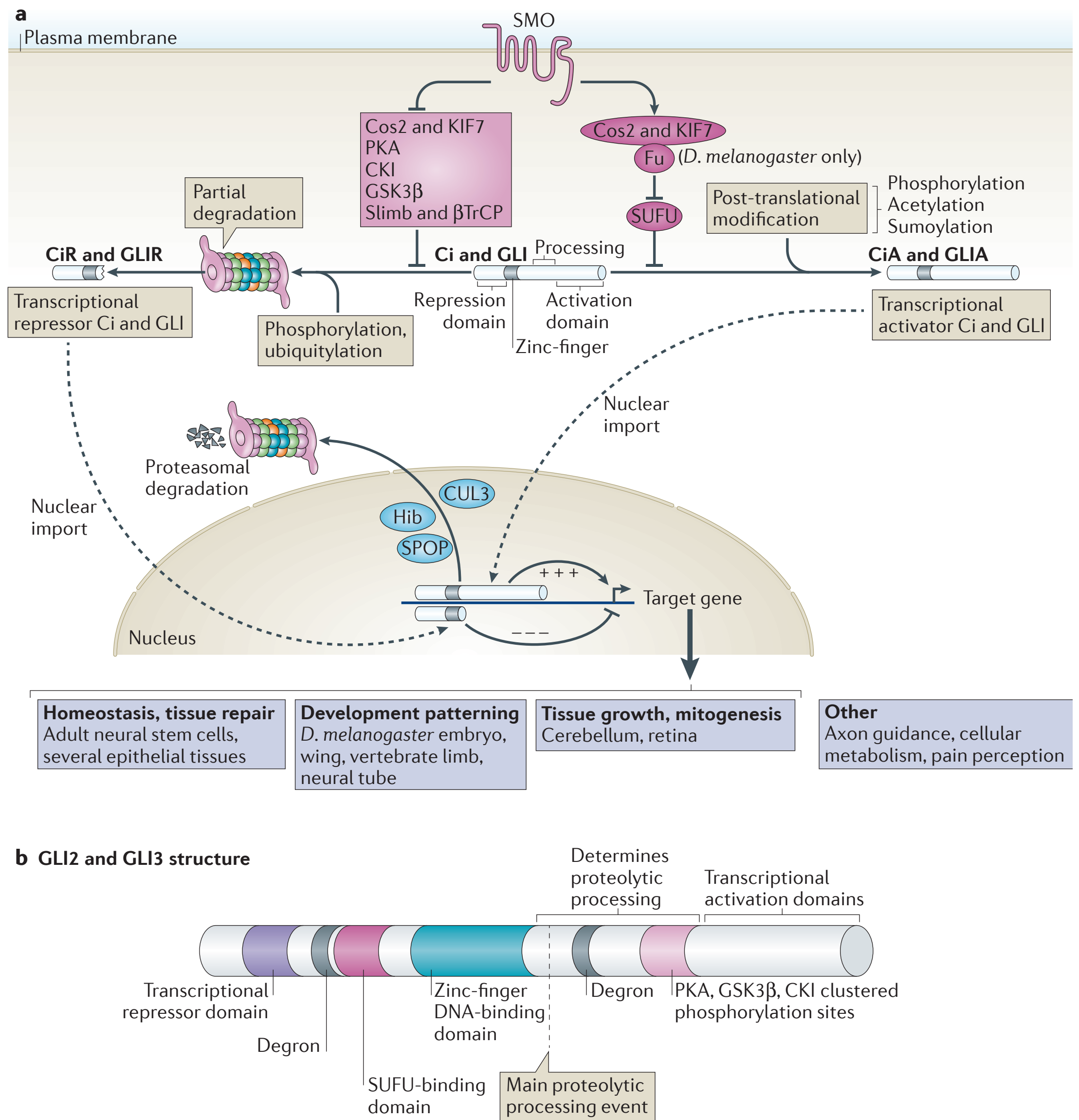

Figure 3 | **Schematic of Ci and GLI regulation.** **a** | In the absence of signal, *Drosophila melanogaster* Cubitus interruptus (Ci) and vertebrate GLI proteins are converted into a transcriptional repressor (CiR and GLIR, respectively). Specifically, the sequential phosphorylation of Ci and GLI proteins by protein kinase A (PKA), CKI and glycogen synthase kinase 3β (GSK3β), which is in part facilitated by Costal 2 (Cos2) and KIF7 in *D. melanogaster* and vertebrates, respectively, promotes the binding of the E3 ubiquitin ligase Slimb and β-transducin repeat-containing protein (βTrCP) and the ubiquitylation of Ci and GLI[107]. The modified protein is then partially proteolytically processed by the proteasome. The truncated protein, which retains its DNA-binding domain, then translocates to the nucleus to repress target gene expression. Activation of Smoothened (SMO) promotes the formation of a transcriptional activator (CiA and GLIA) by blocking Ci and GLI processing and inhibiting the activity of Suppressor of Fu (SUFU; which is a cytoplasmic protein that sequesters Ci and GLI) and Cos2 and KIF7. In *D. melanogaster*, Ci activation depends on the kinase Fused (Fu). In addition to the blockade of Ci and GLI processing, further post-translational modifications, which might include phosphorylation (by unc-51-like kinase 3 (ULK3) or CDC2L1), acetylation or sumoylation, generate the transcriptionally active forms of Ci and GLI proteins (CiA and GliA)[134–137,139]. Inhibition of SUFU activity allows CiA and GLIA to translocate to the nucleus, where they replace CiR and GLIR, respectively, on target genes to activate transcription. Simultaneously, CiA and GLIA become a substrate for proteasomal degradation mediated by Hib (Hedgehog (Hh)-induced BTB protein, in *D. melanogaster*) and SPOP (Speckle-type PDZ protein, in vertebrates) together with cullin 3 (CUL3)[128,130]. HH signalling has pleiotropic functions that include regulating pattern formation in developing tissues, controlling cell proliferation, supporting tissue homeostasis and repair of adult tissues. **b** | A schematic of the domains of GLI2 and GLI3 highlighting the main structural features. These include a DNA-binding domain comprising five C2H2 zinc-fingers, an amino-terminal transcriptional repressor domain and a carboxy-terminal activation domain. In addition, a region C-terminal to the DNA-binding domain contains clusters of phosphorylation sites that are essential for the proteolytic processing event that removes the C-terminal region of the proteins to yield a transcriptional repressor.

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Ci regulation in *D. melanogaster*

Insight into the way in which Smo activity regulates Ci was provided by the identification of a Hh signalling complex (HSC), which interacts with the cytoplasmic C-terminal domain of Smo in *D. melanogaster* and contains Ci and other signalling components[118]. The kinesin-like protein Costal 2 (Cos2)[119] in the HSC serves as a cytosolic scaffold that retains Ci, connecting it to its positive (that is, the Ser/Thr kinase Fused (Fu)) and negative (that is, Pka, Gsk3β and CkI) regulators (FIG. 2a). The kinesin motor function of Cos2 mediates the movement of the HSC along microtubules in cultured cells, at a velocity similar to that achieved with classic kinesins[120]. The role of this movement is not understood, but it may be required to transport Ci and Smo to specialized cellular compartments. In the absence of Hh, HSC-associated kinases promote the partial degradation of Ci to form the transcriptional repressor (CiR)[121]. In the presence of Hh, stabilization and dimerization of phosphorylated Smo at the plasma membrane promotes its association with the HSC and subsequently the autoactivation of Fu, possibly through its dimerization, which in turn phosphorylates Cos2 (REFS 122–125). Different magnitudes of Smo activation are translated into differences in Fu activation, which manifests as different degrees of Cos2 phosphorylation[126]. As a consequence of Cos2 phosphorylation, the uncleaved form of Ci dissociates from the HSC and is able to translocate to the nucleus. Translocation of Ci can also be restrained by its binding to the cytoplasmic protein Sufu (Suppressor of Fu), which is present in large excess[127]. Genetic analysis has shown that the absence of Sufu compensates partially for the absence of Fu, suggesting that Fu is required to antagonize Sufu. How this antagonism operates is unclear as none of the Fu-dependent phosphorylation sites on Sufu identified to date have a significant role[123]. Thus, Fu and other proteins in the HSC may modify Ci directly to prevent its association with Sufu, but this needs to be investigated further. In *D. melanogaster*, the absence of Sufu has no effect on Hh signalling, whereas the absence of Cos2 leads to the ectopic activation of the pathway, suggesting that Cos2 and not Sufu is the major inhibitory regulator of Ci. Once released from Cos2 and Sufu, Ci translocates to the nucleus, however the regulation of the nuclear import of Ci (and GLI proteins) has not been investigated in any detail. In the nucleus, Ci binds to target sites, presumably replacing CiR, and activates gene expression. Notable targets include *ptc*, the upregulation of which confers negative feedback to the pathway. Ultimately, in both *D. melanogaster* and vertebrates, activated Ci or GLI proteins become substrates for CUL3-based E3 ubiquitin ligase SPOP (Speckle-type POZ; the vertebrate homologue of *D. melanogaster* Hib), leading to their degradation[128–131].

Vertebrate GLI proteins and primary cilia

The mechanism of signal transmission between SMO and GLI proteins has diverged between mammals and *D. melanogaster*. In mouse, loss of SUFU results in constitutive activation of the HH pathway[132,133], but the loss of FU (also known as STK36) has no effect on HH signalling[12]. Other kinases and additional post-translational modifications have been suggested to have a role in regulating GLI activity[134–139]. The finding that vertebrate, but not *D. melanogaster*, HH signalling requires the primary cilia of a cell might explain some of the molecular differences between Ci and GLI regulation[140]. Genetic screens and targeted mutations have implicated a host of cilia-associated proteins in vertebrate HH signalling. In addition, several human diseases, collectively termed ciliopathies, that have symptoms characteristic of defective HH signalling, are caused by mutations in components of cilia.

Scrutiny of the HH signalling defects caused by mutations in ciliary proteins revealed that cilia are essential for keeping the pathway inactive, as well as transducing the activating signal. Clues as to why this is have come from studies of the subcellular location of components of the SHH signalling pathway. In unstimulated cells, PTC is observed in cilia and around the ciliary base[141] and, although SMO, GLI2 and GLI3 are not detectable in cilia in the absence of ligand, they seem to transit through it[129,142–144]. The flux of GLI2 and GLI3 through cilia, which depends on the Cos2 orthologue KIF7, is essential for their proteolytic processing into the repressor forms[143,145]. The three kinases —PKA, GSK3β and CKI — implicated in GLI processing are associated with the basal body of primary cilia[104,146–148] (FIG. 2b). Moreover, ACIII and other adenylyl cyclases[149,150], which generate cAMP to activate PKA, are trafficked into cilia by a mechanism that depends on TULP3 (Tubby-related protein 3) and Tectonic 1. These two proteins have also been implicated in HH signalling. Mice lacking either gene exhibit HH signalling defects reminiscent of the defects observed in embryos lacking cilia, and both proteins are observed at the base of cilia, a position that would allow them to gate proteins into the cilium shaft[151]. Recently, GPR161, which increases cAMP levels, has been found to localize to primary cilia in a TULP3-dependent manner. Moreover, loss of GPR161 increases SHH signalling, suggesting a link between PKA activity and SHH signalling[152]. Together with the observation that proteasomes are enriched at basal bodies[153], this suggests that GLI proteins are phosphorylated and processed into their repressor forms as they exit cilia.

HH pathway activation induces changes in cilia composition. PTC[141] and GPR161 (REF. 152) exit the cilia and SMO enters, either via lateral transport from the plasma membrane[102] or directly from an intracellular vesicle[154] (FIG. 2b). The phosphorylated, active form of SMO interacts with β-arrestin and KIF3A, which facilitates its ciliary accumulation[155,156], and SMO forms a complex with the ciliary proteins EVC (Ellis-van Creveld syndrome protein) and EVC2 (REFS 157,158). This results in the accumulation of SMO in a region just distal to the basal body and enables signalling. HH signalling also increases the levels of GLI2, GLI3 and SUFU in cilia, particularly at the tip[129,143,159]. The end result of pathway activation is the dissociation of the GLI–SUFU complex, within the cilia[142,144], and the transport of the newly liberated full-length, activated GLI2 and GLI3 proteins from the cilia to the nucleus bypassing proteolytic processing.

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Despite this wealth of observations, a coherent molecular mechanism by which a signal is transduced within the cilium remains elusive. Accumulation of SMO in the cilium is not sufficient to transduce the normal intracellular signal, as cyclopamine (a small-molecule antagonist of SMO) promotes the translocation of SMO to cilia but blocks GLI activation[160]. Phosphorylation and a conformational change in the C-terminal tail of SMO is important[105], and this seems to promote an interaction with EVC and EVC2 (REF. 158), but how this is coupled to post-translational changes in GLI proteins is unclear. Furthermore, how components of the signalling pathway are gated into the cilium is unclear, and what modifications or interactions are responsible for regulating their function has not been deciphered. Equally intriguingly, the evolutionary origin of the role of cilia remains unclear. In planarians, cilia do not seem to be required for HH signalling, nevertheless, orthologues of FU and COS2 are present[161]. Thus, the most parsimonious interpretation is that the link between cilia and HH signalling was present in the bilaterian ancestor and this was then adapted and remodelled in distinct lineages.

Functions of HH signalling

HH signalling has vital and diverse roles throughout animal development and adult tissue homeostasis. Here, we summarize some of the themes that connect these diverse functions.

Graded HH signalling and developmental pattern formation. HH acts as a long-range morphogen to control cell patterning and differentiation in several embryonic tissues[107]. Examples include the pattern of venation in *D. melanogaster* wing discs, the digit pattern in vertebrate limbs and the organization of neuronal subtype identity in the vertebrate central nervous system (CNS). In each case, Ci and GLI activity results in the induction of a very similar core set of target genes[162–164], including genes encoding Ptc in *D. melanogaster* and in vertebrates PTC, Patched 2, HIP1 and GLI1, all of which participate directly in the pathway, either influencing the spread of the ligand or downstream signalling. However, diverse cellular responses are seen in different tissues owing to the regulation of different sets of target genes in response to gradients of HH ligands. Thus, in the wing disc, a gradient of Hh induces several genes, including *decapentaplegic* (*dpp*), *collier* and *engrailed*, at different thresholds[165], whereas in the neural tube the genes encoding the transcription factors NK6 homeobox 1 (NKX6.1), oligodendrocyte transcription factor 2 (OLIG2) and NKX2.2 are induced by progressively higher concentrations of SHH[166]. There is evidence that different concentrations of ligand are transformed into different levels of intracellular signalling[99,126], resulting in a Ci and GLI activity gradient[167]. As the activation of some genes requires a greater change in the repressor to activator ratios of Ci and GLI proteins than others[168–170], a gradient of Ci and GLI activity could contribute to tissue patterning activity. However, studies in several tissues indicate that the duration of HH signalling is also important[171–173]. This has led to a model in which Ci and GLI activity, which is controlled by HH signalling, acts as a component of the gene regulatory networks responsible for patterning the particular tissue, and this allows both the level and the timing of Ci and GLI activity to influence when and where genes are activated[174].

Chromatin immunoprecipitation studies in neural and limb cells, using epitope-tagged versions of GLI1 or GLI3 in cells responding to SHH signalling, have revealed several thousand genomic binding sites for these proteins[162–164,175]. Most, but not all, of these contain a motif similar to the previously defined Ci- and GLI-binding consensus sequence GACCACCCA[176]. As expected, GLI binding is observed around the genomic areas encoding core targets of the pathway, such as PTC, in cells from each of the tissues analysed. More strikingly, many of the targets that are regulated exclusively in one tissue are also bound by GLI proteins in the other tissue, suggesting that GLI binding alone does not explain specificity of regulation[162]. In addition, functional tests in the neural tube identified GLI-binding enhancer elements acting as positive regulators of gene expression that require SHH signalling for their activation, whereas other GLI-binding elements act as inhibitors of gene expression and require the removal of GLI repressor activity for gene induction[170]. This is in line with the differential sensitivity of genes to the ratio of repressor Ci and GLI proteins to activator Ci and GLI proteins. It is also consistent with the observation that, in mouse embryos lacking SHH, the removal of GLI3, which provides most of the transcriptional repressor activity, recovers the expression of some target genes[111]. Thus, some SHH target genes require positive input from a GLI transactivator to initiate transcription, whereas others are activated by removing the GLI repressor protein from the enhancer. The mechanism that determines these different responses to GLI proteins remains to be determined.

The role of HH signalling in various developing tissues explains why genetic defects in the pathway lead to several severe congenital abnormalities[10]. In addition to ciliopathies, HH pathway defects can cause holoprosencephaly, which results from an incomplete cleavage of the forebrain along the ventral midline, or complex genetic diseases, such as Pallister–Hall syndrome, that involve polydactyly (additional fingers or toes) and other organ defects. Moreover, the involvement of IHH in the development of the endochondral skeleton of vertebrates can explain the relationship between HH signalling and the regulation of adult height[177] and why point mutations in *IHH* cause brachydactyly (truncated limb digits)[178].

HH acts in networks of secreted factors. HH signalling frequently induces the expression of additional secreted molecules to produce interacting signalling networks. For example, the hedgehog like appearance of *D. melanogaster* embryos lacking Hh is a consequence of a loss of reciprocal feedback between adjacent stripes of cells that normally express Wingless (Wg) and Hh[179]. This reciprocal positive feedback between Wg and Hh maintains the expression of the two ligands in stripes along the anterior–posterior axis and elaborates the segmental development of the embryo. Similarly, during bladder regeneration in vertebrates, cells within the urothelium

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secrete SHH to induce the expression of WNT proteins in the adjacent stromal cell layer, which in turn signal back to the urothelium[180].

In addition to WNT, HH partners with other signalling molecules. In the *D. melanogaster* wing disc, Hh secreted from the posterior half of the disc induces the bone morphogenetic protein (BMP) family protein Dpp, in a stripe in the middle of the disc, which then controls patterning and growth of the nascent wing blade[181]. During vertebrate limb development, a set of linked feedback loops, involving SHH, BMP and the BMP antagonist gremlin 1 (GREM1), are responsible for limb outgrowth and patterning[182]. In this case, SHH signalling maintains the expression of GREM1 to limit BMP signalling. An analogous signalling loop between SHH and BMP antagonists has been proposed to contribute to the elaboration of hair follicles in the skin[183]. Just as for SHH and WNT in the bladder, this involves reciprocal feedback between SHH-expressing cells in the epithelial layer and BMP-expressing cells in the mesenchymal layer. Similar examples of HH proteins participating in networks of signalling molecules that maintain and elaborate tissue architecture can be found in many tissues. Moreover, evidence of intracellular crosstalk between the HH pathway and other signalling pathways also adds to the complexity in understanding the interactions between the pathways (for a review, see REF. 184).

HH signalling in stem cells and cancer. Together with its function as a developmental morphogen, HH regulates the survival and proliferation of several tissue progenitor and stem populations[11]. During cerebellum development, SHH secreted by Purkinje cells supports the proliferation of granule cell precursors in the external granular layer[185] by promoting the expression of several well-known stem cell and proliferative genes, including genes encoding MYC, cyclin D1, insulin-like growth factor 2 (IGF2) and BMI1 (REF. 186). In the adult brain, HH signalling maintains neural stem cells that continuously supply new neurons[187,188]. HH signalling is also required for the maintenance of stem cells in a range of tissues, from the hair follicle to the haematopoetic system, and it is also involved in the injury-dependent regeneration of many organs, including the exocrine pancreas, prostate and bladder[11]. Whether there are common molecular mechanisms that link these mitogenic and survival roles of HH remains to be seen.

The influence of HH signalling on stem cells suggests why activation of the pathway has been found in various human tumours[189]. The heritable condition Gorlin's syndrome is caused by heterozygousity in *PTC*[190,191] and is characterized by a high incidence of the skin cancer basal cell carcinoma (BCC) and the cerebellum cancer medulloblastoma. Moreover, sporadic occurrences of BCC and a subset of medulloblastomas are the result of *de novo* mutational activation of the pathway[189]. In both BCCs and medulloblastomas, dysregulation of HH signalling in a stem cell and precursor cell population, respectively, seems to explain tumour formation. For medulloblastomas, the granular cell precursors in the neonatal cerebellum are the cells of origin and this explains the childhood bias of this tumour[192]. For BCCs the origin seems to be either stem cells within the hair follicle[193] or in the interfollicular epidermis[194]. The recent regulatory approval of a drug that blocks SMO activity has raised the prospect that these tumours may now be amenable to treatment[195].

In contrast to these ligand-independent tumours, many of the other tumours associated with HH pathway activation do not contain mutations in pathway components. Instead, pathway activation depends on ligand production, either by the tumour cells themselves or the surrounding stroma[189,196]. Two hypotheses, which are not mutually exclusive, have been suggested to explain the role of HH signalling in these tumours. First, the survival or proliferation of tumour cells directly requires HH signalling. In support of this, *in vitro* studies have demonstrated that HH signalling supports and enhances cancer cell growth[197]. *In vivo* evidence in support of this autocrine and juxtacrine mechanism is currently more limited and in some cases contentious[198], although the recent observation that HH signalling promotes Warburg-like glycolytic metabolism[199], which is found in many tumours, suggests a possible role for signalling within the tumour cells. As an alternative, however, HH production has been hypothesized to promote the tumour microenvironment in a paracrine manner by signalling to the stroma, which then signals back to the tumour[198]. This could be analogous to the reciprocal signalling networks that HH establishes during embryonic development. The exact function of HH signalling in these tumours, and whether it represents a dysregulation of the normal stem cell maintenance and regeneration functions of HH signalling, remains to be determined. Disappointingly, however, recent clinical trials of drugs that target the HH pathway in patients with tumours classed as ligand-dependent have been discouraging, suggesting that we still have much to learn about these cancers. Resolving the importance of HH signalling, and whether it acts autonomously or non-autonomously in ligand-dependent tumours, is crucial as these cancers affect many more individuals than those with BCCs and medulloblastomas.

Non-canonical HH signalling. The functions described above represent only a few examples of the roles ascribed to HH signalling. The list continues to grow, for instance, HH signalling has recently been shown to regulate pain perception[200] and cellular metabolism[199]. Several of the non-canonical roles of HH signalling seem to depend on HH signalling via alternative, non-transcriptional mechanisms. Among these, HH signalling in axon and cell chemotaxis are the best described. The projection of spinal commissural axons towards the ventral midline requires SHH that is produced in the floor plate and depends on SHH signalling through PTC and SMO but not on a transcriptional response[201,202]. Instead, SMO-dependent activation of SRC family kinases has been implicated[203]. Once across the midline, the expression of HIP1 in commissural spinal neurons has been proposed to participate in their anterior routing, independently of SMO[202]. SHH also guides axons of retinal ganglion cells in the visual system[204,205].

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Box 2 | **Ten unanswered questions on Hedgehog signalling and its physiological roles**

- In what form(s), and by what route, does Hedgehog (HH) spread through tissues?
- What is the composition of the HH receptor complex(es)?
- How does Patched 1 (PTC) regulate Smoothened (SMO)?
- How is the signal transduced from SMO to GLI and what controls the subcellular localization of GLI proteins and *D. melanogaster* Cubitus interruptus (Ci)?
- What is the function of cilia and proteins localized at the cilia in vertebrate HH signalling?
- What is the evolutionary origin of the role of cilia in the HH pathway and/or how was this function lost?
- What non-canonical mechanisms of HH signalling are activated by the pathway?
- What functions does HH signalling have in addition to its roles in tissue development and homeostasis?
- What role does HH signalling have in cancers other than basal cell carcinoma and medulloblastoma?
- What is the mechanism of gene regulation by GLI proteins and how are cell type-specific responses determined?

Chemotaxis of fibroblasts induced by SHH signalling also seems to be independent of transcription[206,207]. Recent studies have suggested that this process does not require cilia. Instead, the small RHO GTPases RAC1 and RHOA, which are activated by SMO in a Gi-dependent fashion, have been implicated[208]. The activation of Gi proteins and downstream phospholipase Cγ (PLCγ) might also account for the Ca^{2+} spikes observed in neural progenitors in response to SHH[209]. Intriguingly, a recent report indicates that cyclopamine, which blocks canonical signalling from SMO to GLI proteins, is sufficient to mobilize Ca^{2+}, suggesting that cyclopamine acts as a partial and selective agonist for SMO signalling[199]. Finally, PTC has been proposed to act as a 'dependence receptor' that induces apoptosis in the absence of SHH. This seems to be the result of PTC interacting with a protein complex, including down-regulated in rhabdomyosarcoma LIM (DRAL) and caspase 9, which triggers a caspase-dependent cell death pathway[210,211]. Clearly more work is required to elucidate the various proposed non-canonical signalling pathways and to understand their contribution to HH responses.

Outlook

Taken as a whole, the past 20 years of study has revealed a wealth of detail and an unexpected diversity in the mechanisms and functions of HH signalling. The identification of the ligands and core components of the pathway in different branches of the bilaterian family emphasizes its importance and deep evolutionary origin. The conserved but unusual way in which HH ligands are generated and released from cells represented a great surprise. Moreover, the relatively recent finding that cilia are involved in vertebrate HH signalling but not in *D. melanogaster* has added a new twist to the field and revitalized interest in this previously ignored organelle. Despite the advances, however, there are still large gaps in our knowledge of HH signalling and many unanswered questions (BOX 2). In some cases it seems likely that new tools and reagents will provide new insight, perhaps the increasing power of proteomic and metabolomic techniques will help address how PTC regulates SMO and the role of cilia in this process and in the downstream transduction of the signal. For other questions, such as the function of HH signalling in tissue development and stem cells, dissecting the complex gene networks that are involved and searching for commonalities between the networks in different cell types is likely to yield conceptual insight. It is perhaps the importance of these outstanding issues that say most about the pathway. Addressing these issues is crucial for understanding this fascinating signalling pathway and for developing therapeutic strategies to target the medical consequences of its dysregulation. Furthermore, given the surprises this pathway has delivered over the past two decades, who knows what secrets are yet to be revealed.

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