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DEVELOPMENT AT A GLANCE

Hedgehog signalling

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ABSTRACT

The Hedgehog (Hh) signalling pathway is one of the key regulators of metazoan development. Hh proteins have been shown to play roles in many developmental processes and have become paradigms for classical morphogens. Dysfunction of the Hh pathway underlies a number of human developmental abnormalities and diseases, making it an important therapeutic target. Interest in Hh signalling thus extends across many fields, from evo-devo to cancer research and regenerative medicine. Here, and in the accompanying poster, we provide an outline of the current understanding of Hh signalling mechanisms, highlighting the similarities and differences between species.

KEY WORDS: *Drosophila*, Hedgehog signalling, Vertebrate

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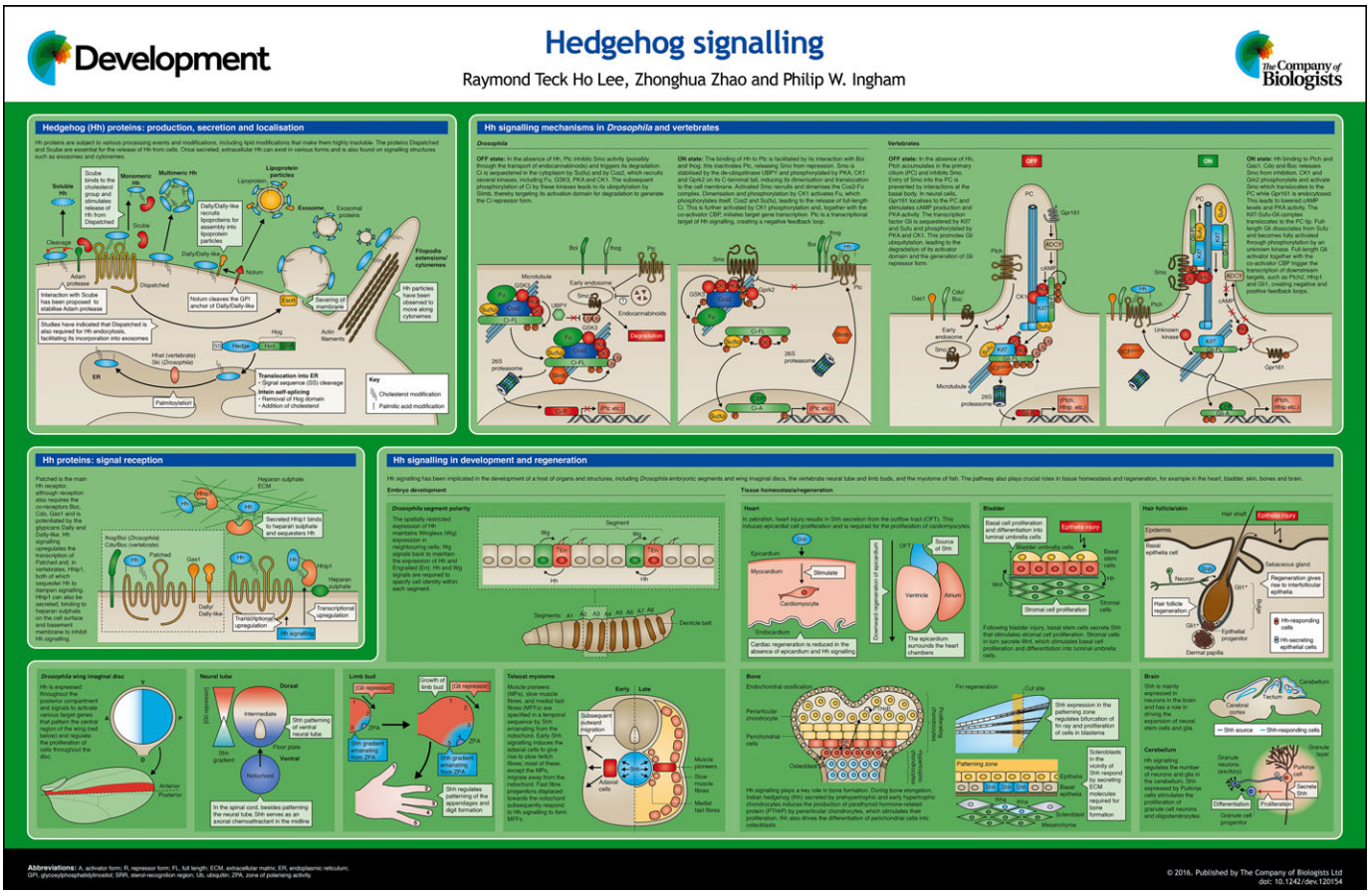
Introduction

Hedgehog (Hh) signalling was initially characterised through its role in patterning the *Drosophila* larval epidermis, where spatially restricted expression of Hh – the single Hh family protein present in flies – maintains expression of the *Drosophila Wnt1* orthologue *wingless* (*wg*) in neighbouring cells (Ingham and Placzek, 2006). Subsequently, a combination of gain- and loss-of-function studies focusing largely on the wing uncovered a role for Hh signalling in patterning the appendages of the adult fly (Hartl and Scott, 2014). Hh is expressed throughout the posterior compartment of the wing imaginal disc and signals across the compartment boundary to activate various target genes that regulate the proliferation and positional identity of cells throughout the disc.

In vertebrates, the spatially restricted expression of sonic hedgehog (Shh) – the most extensively studied of the vertebrate Hh proteins – in the developing limb bud plays a remarkably similar role, regulating cell proliferation and patterning of the appendages (Ingham and Placzek, 2006). Recent studies have also revealed how modulation of the response to Shh underlies interspecies digit variation (Lopez-Rios et al., 2014). A second well-characterised role of Shh is in the specification of cell types within the neural tube.



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In this context, different levels and duration of exposure to Shh combine to allocate cells to unique neuronal identities (Dessaud et al., 2008). Besides these classic examples, Hh signalling has been implicated in the development of a multitude of other organs and structures, including the adrenal cortex, cerebellum, eye, face, gastrointestinal tract, hair follicle, kidney, long bone, lung, pancreas, pituitary gland, prostate, skeletal muscle and teeth (McMahon et al., 2003). In addition, Shh functions as an axon guidance cue (Yam and Charron, 2013) and plays a crucial role in tissue homeostasis as well as in the regenerative response to injury in a number of organs, including the bladder, airway, heart and fin (Bellusci et al., 1997; Shin et al., 2011; Wang et al., 2015; Wehner and Weidinger, 2015).

Hh proteins and their secretion

Hh proteins are found in many animals, from jellyfish to human (Ingham et al., 2011). *Drosophila* has only one *hh* gene, whereas vertebrates have between three and five. All Hh proteins are composed of a so-called N-terminal 'Hedge' domain and a C-terminal 'Hog' domain. The Hedge domain mediates the signalling activity of the protein. The Hog domain can be further subdivided into an N-terminal Hint domain, which is similar in sequence to self-splicing inteins, and a C-terminal sterol-recognition region (SRR) that binds cholesterol (Ingham et al., 2011). The Hint domain promotes the cleavage of full-length Hh in the endoplasmic reticulum, which results in the covalent coupling of a cholesterol moiety to its C-terminus (Briscoe and Thérond, 2013). Subsequently, the N-terminus of cleaved Hh protein becomes palmitoylated, a modification essential for Hh activity. This dual lipidation renders Hh proteins highly hydrophobic, a property expected to cause their retention in the plasma membrane. This raises important questions about the way in which Hh proteins can signal over many cell diameters, as implied by their well-established roles as morphogens (Briscoe and Thérond, 2013).

These questions have been addressed most extensively by experiments in *Drosophila*, where sophisticated genetic manipulations allow analyses in an *in vivo* context, and have been complemented by biochemical studies undertaken largely in mammalian tissue culture systems. For example, the analysis of a number of *Drosophila* mutants has provided insight into the factors involved in the release and movement of lipidated Hh protein. *dispatched* (*disp*) encodes a multipass transmembrane protein homologous to the Hh receptor Patched (see below) that is required for the release of Hh from Hh-producing cells. The *dally* and *dally-like* genes [glypican (Glp) family in vertebrates] encode core proteins of heparan sulphate proteoglycans, the synthesis of which requires the glycosyltransferase encoded by *tout velou* [*ttv*; exostosin glycosyltransferase (Ext) genes in vertebrates]. Genetic analysis has implicated the products of these genes in the reception and movement of Hh by responding cells, as well as in its secretion by producing cells (Briscoe and Thérond, 2013). Genetic analysis in mouse has confirmed a role for the Disp orthologue Disp1 in the secretion of vertebrate Hh proteins. A direct interaction between Disp1 and the cholesterol moiety of the Hh ligand has been proposed to facilitate transfer of Hh protein to the outer leaflet of the plasma membrane, from which it is subsequently released through a 'hand-off' process involving displacement of cholesterol binding from Disp1 to the secreted Scube2 protein. Other studies, however, have suggested that the release of Hh from secreting cells is driven instead by a 'shedding' reaction involving the cleavage of both its N-terminal and C-terminal ends by ADAM proteases, thus releasing an unlipidated soluble form of Hh from the cell (Briscoe and Thérond, 2013). According to this view, Scube2 and the glypicans contribute to Hh release by potentiating the activity of the ADAM proteases involved in shedding (Jakobs et al., 2014; Ortmann et al., 2015).

Unlipidated active forms of Hh have been reported to be secreted by *Drosophila* and mammalian cells; however, there is good evidence that lipidated Hh forms are also released by cells, as monomers or multimers, or in association with lipoproteins or exosomes (Briscoe and Thérond, 2013; Gradilla et al., 2014; Matusek et al., 2014). The importance of exosomes for Hh signalling is further supported by recent findings in *Drosophila* demonstrating that ESCRT (endosomal sorting complex required for transport) proteins are necessary for Hh release and long-range signalling activity. In line with this, Hh is found in association with ESCRT proteins in exosome-like vesicles. Remarkably, Disp has been implicated in this process, not through promoting the release of Hh but rather through promoting its endocytosis by secreting cells, which is thought, in turn, to facilitate the packaging of Hh into exosomes (D'Angelo et al., 2015). Other studies have similarly implicated Disp in the recycling of Hh protein by secreting cells, a process that has also been proposed to facilitate the loading of Hh-containing exosomes into cytonemes (Briscoe and Thérond, 2013), long filopodia-like extensions that have been implicated in the transport and activity of a number of signalling proteins (Kornberg and Roy, 2014). Structures similar to cytonemes have also been implicated in Hh signalling in the chicken limb bud (Sanders et al., 2013).

Hh signal reception

Genetic analyses in *Drosophila* originally identified the multipass transmembrane protein encoded by the segment polarity gene *patched* (*ptc*) as the Hh receptor (Ingham and McMahon, 2001). Vertebrates have two Patched genes (*Ptch1* and *Ptch2*), the products of which play partially redundant roles in Hh reception. The binding of Hh proteins to Ptch proteins promotes pathway activation by inhibiting the function of the Ptc/Ptch receptor. In addition, Hh proteins bind to several other type 1 membrane proteins that are required for pathway activation: vertebrate Cdo and Boc and their *Drosophila* counterparts Ihog and Boi, and the vertebrate-specific Gas1. These co-receptors act semi-redundantly to mediate Hh pathway activation. Accordingly, mice and flies mutant for only one gene show mild defects in Hh signalling, whereas the simultaneous inactivation of Ihog and Boi in flies (Beachy et al., 2010), or of Cdo (Cdon), Boc and Gas1 in mice (Allen et al., 2011), leads to a complete loss of pathway activation. In mice, the binding of Hh ligand to Ptch is mediated by its interaction with co-receptors, whereas in flies, Hh can bind to Ptc independently of Ihog and Boi, neither of which can bind Hh independently of Ptc (Beachy et al., 2010).

Ptc/Ptch proteins also serve to limit Hh signalling by sequestering and endocytosing Hh ligand, a function also fulfilled in vertebrates by Hh interacting protein 1 (Hhip1) (Ingham and McMahon, 2001). The expression of Ptc/Ptch and Hhip1 is induced upon Hh pathway activation, creating a negative-feedback loop. In contrast to Ptc/Ptch, Hhip1 also exists in a secreted form that inhibits Hh signalling non-autonomously (Holtz et al., 2015).

Hh signal transduction in *Drosophila*

A notable and unusual feature of the Hh signal transduction pathway is its induction by receptor inactivation. This first became apparent through the finding that Hh target genes are expressed ectopically in *Drosophila* embryos that lack Ptc activity, even in the absence of the

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Hh ligand. This reflects the role of Ptc in suppressing the activity of Smoothened (Smo), a member of the G-protein coupled receptor (GPCR) superfamily, most closely related to the Frizzled (Fz) family of Wnt receptors. Smo is an obligate transducer of the Hh signal; its inactivation causes the complete loss of Hh target gene expression, even in the absence of Ptc (Ingham and McMahon, 2001). Exactly how Ptc regulates Smo activity is still unclear, but its homology to the cholesterol transporter Npc1 has prompted the view that it may traffic lipid agonists or antagonists of Smo (Incardona et al., 2002). Interestingly, the binding of oxysterols to the extracellular N-terminal domain of Smo is sufficient to activate the Hh pathway (Nachtergaele et al., 2013), although there is no evidence that oxysterols are endogenous Smo ligands. Furthermore, endocannabinoids have recently been identified as putative Smo inhibitors that are carried into the cell on liposomes and recruited by Ptc into endosomes, where they are thought to bind directly to Smo to inhibit its activity (Khaliullina et al., 2015). Ptc has also been implicated in regulating Smo activity by modulating phosphoinositiol levels in *Drosophila*; the loss of Ptc thus results in increased levels of PI4P, which in turn promotes Smo activation (Yavari et al., 2010).

When activated, Smo accumulates at the plasma membrane, where it becomes hyperphosphorylated through the coordinated activities of Protein kinase A (PKA), Casein kinase 1 (CK1), Glycogen synthase kinase 3 (GSK3; Shaggy – FlyBase) and G-protein coupled receptor kinase 2 (Gprk2) (Su et al., 2011). Phosphorylation occurs on multiple sites in the Smo intracellular C-terminal tail, leading to a conformational change that recruits the kinesin family protein Costal-2 (Cos2; Costa – FlyBase) and promotes its dimerisation (Shi et al., 2011). This in turn leads to the dimerisation and activation of Fused (Fu), a serine-threonine kinase that binds to Cos2 (Shi et al., 2011). Fu activity is essential for transduction of the Hh signal, and its absence causes a loss of Hh target gene activation (Zhou and Kalderon, 2011). This effect can, however, be suppressed by mutation of the *Suppressor of fused* [*Su(fu)*] gene (Ingham and McMahon, 2001), the product of which is homologous to bacterial proteins of unknown function (Ingham et al., 2011). The main role of Su(fu) appears to be to repress the transcription of Hh target genes by binding to the zinc finger transcription factor Cubitus interruptus (Ci) and preventing its nuclear import, although Su(fu) can also enter the nucleus to modulate transcription (Cheng and Bishop, 2002). Remarkably, despite this apparently crucial function, Su(fu) is completely dispensable for normal development in *Drosophila*, its loss having no discernible effect on Hh pathway activity (Ingham and McMahon, 2001). This reflects the fact that Cos2 has a similar restraining effect on Ci, binding the transcription factor and retaining it in the cytoplasm in the absence of Smo activation (Wang and Jiang, 2004). This dual role of Cos2 is crucial for normal operation of the Hh signal transduction pathway: in the absence of Hh signal, Cos2 also recruits PKA, CK1 and GSK3, bringing them into close proximity with the Ci protein (Zhang et al., 2005). Ci is a bifunctional transcription factor, containing both repressor and activator domains that flank the centrally located zinc finger DNA-binding domain (Ingham and McMahon, 2001). Recruitment of PKA/CK1/GSK3 to the Cos2-Ci complex results in the phosphorylation of Ci on multiple sites in its C-terminus; the phosphorylated residues are recognised by the F-box protein Slmb, the binding of which promotes Ci ubiquitylation, targeting it for partial proteolysis by the proteasome (Ingham and McMahon, 2001). The resultant truncated form of Ci (Ci-R), which lacks the C-terminal activator domain of the protein but retains the N-terminal repressor domain, enters the nucleus and represses the transcription of Hh target genes (Ingham and McMahon, 2001). The binding of Cos2 to the C-terminal tail of activated Smo disrupts Ci phosphorylation by promoting PKA recruitment to the plasma membrane, thereby abrogating Ci cleavage and allowing the full-length form of Ci (Ci-A) to accumulate, enter the nucleus and activate Hh target genes (Zhang et al., 2005). The phosphorylation of full-length Ci is thought to be necessary for its activity. In addition, Fu activation facilitates the nuclear import of Ci by promoting its dissociation from Su(fu) (Zhou and Kalderon, 2011).

Hh signal transduction in vertebrates

Vertebrates share most of the core components of the Hh signalling pathway with *Drosophila*, and their mechanisms of Hh signal transduction show some degree of similarity (Ingham et al., 2011). As in *Drosophila*, vertebrate Hh ligands bind to and inactivate the Ptch proteins, which in turn act to inhibit Smo. Downstream of Smo, the vertebrate Cos2 orthologue Kif7 acts together with Sufu to restrain the transcriptional response to Hh. There are, however, several notable differences between the phyla.

First, there has been duplication of genes encoding several components of the Hh pathway in vertebrates. This includes not only the *Hh* gene itself, of which there are three paralogues in birds and mammals (Desert, Indian and Sonic), two of which (Indian and Sonic) are duplicated in fish, but also the Ptch gene (of which there are two paralogues in all vertebrates) and the gene encoding the transcription factor Ci, the functions of which are distributed between three Gli family proteins in birds and mammals (Gli1-3), with an additional Gli2 paralogue in fish. Like Ci, the Gli2 and Gli3 proteins contain both activator and repressor domains and undergo proteasome-dependent proteolytic cleavage. As in *Drosophila*, this is promoted by PKA activity and transforms the transcription factors from activators into repressors (Pan et al., 2009); Gli3, however, acts mainly as a repressor, whereas Gli2 appears to function mostly as an activator. Gli1, by contrast, lacks the N-terminal repressor domain and functions exclusively as an activator. The *Gli1* gene is also a target of Hh signalling and thus acts to amplify the response to the signal (Ingham et al., 2011).

A more remarkable difference is in the requirement for the Sufu protein; whereas in *Drosophila*, Su(fu) loss has no obvious effect, except when activity of the kinase Fu is impaired, Sufu loss in mammals causes a major derepression of the Hh pathway, resulting in embryonic lethality (Svärd et al., 2006). This seems to reflect a major role for Sufu in maintaining the Gli transcription factors in an inactive state by physically associating with them, a role that in *Drosophila* is fulfilled principally by the Cos2 protein (Merchant et al., 2004). More remarkable still is the absence of a role for Fu in transducing the Hh signal in mammals. Whereas loss of Fu activity in *Drosophila* results in the failure to activate Hh target genes, mice homozygous for mutations of the orthologous *Stk36* kinase show no defects in their Hh response (Chen et al., 2005). Exactly when this divergence occurred remains unclear; several studies have implicated Stk36 in Hh signalling in zebrafish (Ingham et al., 2011), but a definitive analysis of its role based on mutations, rather than antisense knockdown, has yet to be undertaken.

Perhaps the most striking difference between Hh signalling in *Drosophila* and vertebrates, however, is the dependence of the latter on the primary cilium (PC). This primitive organelle was first implicated in Hh signalling through the isolation of mouse mutations disrupting primary ciliogenesis that have phenotypes indicative of aberrant Hh signalling (Huangfu et al., 2003). Subsequent analyses in chick and fish revealed a universal

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involvement of the PC in Hh signalling across vertebrates (Huang and Schier, 2009; Yin et al., 2009). The central role of the PC in transduction of the Hh signal is reflected in the dynamic localisation of the various Hh pathway components to this organelle. In the absence of ligand, the Hh receptor Ptch accumulates in and around the PC, but on exposure to ligand it dissipates to be replaced by Smo (Rohatgi et al., 2007), which moves into the PC by lateral transport (Milenkovic et al., 2015; Ye et al., 2013). The localisation of Smo to the PC is a necessary, although not sufficient, step in its activation, in response to which the Gli transcription factors, complexed with Sufu, are transported to the tip of the PC (Haycraft et al., 2005). This translocation appears to be essential for dissociation of the Gli-Sufu complex and hence for Gli activation (Tukachinsky et al., 2010); in line with this, mutations that disrupt the integrity of the PC render cells unresponsive to ligand-mediated pathway activation (Goetz and Anderson, 2010). However, the PC is also crucial for the generation of the repressor forms of Gli factors, acting as a centre for the localised production of cAMP and the concomitant activation of PKA. In neural cells, for instance, the GPCR Gpr161 localises to the PC in the absence of Hh signal and activates adenylcyclase through the Gαs G-protein (Mukhopadhyay et al., 2013). Accordingly, loss of the PC also disrupts the PKA-dependent proteolytic cleavage of Gli factors as well as their activation. Consequently, animals mutant for genes that encode proteins involved in the formation or maintenance of the PC, such as the centriolar Talpid3 protein (Yin et al., 2009) and intraflagellar transport (IFT) proteins (Goetz and Anderson, 2010), display both gain and loss of Hh function phenotypes, reflecting the differing contributions of Gli-mediated repression and activation to the specification of different organs.

The vertebrate orthologue of Cos2, Kif7, also localises to the PC, where it has been implicated in the anterograde transport of Gli proteins in response to Shh activity (Ingham and McMahon, 2009). In the absence of ligand, Kif7 also accumulates in cytoplasmic puncta that dissipate in response to pathway activation (Maurya et al., 2013). Like Cos2, Kif7 acts downstream of Smo, both to restrain and potentiate Gli activity. The former effect is thought to reflect the promotion of Gli cleavage by Kif7 (Ingham and McMahon, 2009); however, Kif7 also functions to restrain the activity of Gli1, at least in zebrafish, (Maurya et al., 2013). Potentiation of the pathway is achieved by promoting the dissociation of Gli proteins from Sufu (Maurya et al., 2013). In addition, Kif7 has been implicated in limiting the length of the PC through binding at microtubule plus ends and by promoting microtubule catastrophe (He et al., 2014). Exactly how this impacts on the regulation of Gli activity is currently unclear.

Non-canonical Hh signalling

As well as signalling via the 'canonical' pathway outlined in the preceding sections, a growing number of reports have suggested that Hh proteins can elicit effects via other, 'non-canonical' pathways. The fact that Smo belongs to the GPCR superfamily has long prompted the notion that it might signal like other classical GPCRs via heterotrimeric G-proteins. Several lines of evidence have been presented in support of this view: in *Drosophila*, genetic ablation of Gαi activity in imaginal disc cells was found to cause downregulation of the Hh target gene *dpp*, whereas expression of a constitutively active form of Gαi rescued the effects of dominant-negative inhibitors of Smo activity (Ogden et al., 2008). Although these findings, together with the demonstration that pertussis toxin, which is a potent inhibitor of Gi proteins, can suppress Hh target gene expression in mammalian cells (Riobo et al., 2006) and zebrafish embryos (Hammerschmidt et al., 1996), support a connection between Smo and Gαi activity, they cannot alone distinguish between Smo-dependent regulation of Gαi and Smo-independent modulation of PKA activity by Gαi. Consistent with the former scenario, the reduction in cAMP levels by Hh was found to be sensitive to both Gαi and Smo knockdown in insect cells (Ogden et al., 2008). Furthermore, using a classic assay for G-protein coupling, the binding of isotopically labelled GTP to Gi family proteins was shown to be stimulated by Smo in insect (Sf9) and mammalian [mouse embryonic fibroblast (MEF)] cells (Riobo et al., 2006). Definitive *in vivo* evidence that Smo acts directly through G-proteins to mediate the transcriptional response to Hh, however, remains elusive.

In other contexts, Hh proteins have been shown to elicit effects independently of the Gli transcription factors. The role of Shh as an axon guidance signal in vertebrates provides perhaps the best-characterised example of such non-canonical signalling. In this context, commissural axons respond to Shh exposure within a few minutes by activating the Src family kinase (SFK) in a Smo-dependent, but transcription-independent, manner (Yam et al., 2009). Shh-induced stress fibre formation in endothelial cells and fibroblasts, as well as dendritic spine formation in hippocampal pyramidal neurons, also appear to be mediated independently of transcriptional activation by Smo-dependent activation of RhoA and Rac1 (Brennan et al., 2012).

Shh signalling has also been implicated in the metabolic reprogramming of adipocytes and MEFs in a transcription-independent manner (Teperino et al., 2012). Activation of Smo in these cells results in a rapid increase in glucose uptake, an effect mediated through the activation of AMP-activated protein kinase (AMPK). This response is sensitive to pertussis toxin, implying that it is also regulated via Gαi activity. Notably, cyclopamine, which effectively inhibits canonical signalling by binding to Smo, acts as an agonist of this non-canonical pathway, indicating that Smo can independently regulate two distinct downstream effectors (Teperino et al., 2012).

Several reports have also identified processes that are regulated by Ptc independently of Smo, suggesting various roles for Ptc. The most extensively studied of these is the proposed role of Ptc as a dependence receptor (Brennan et al., 2012). According to these analyses, the activity of Shh as a survival factor is mediated by opposing the apoptosis-promoting activity of Ptc. Indeed, in the absence of Shh ligand, Ptc1 overexpression induces apoptosis both *ex vivo* and *in vitro*, an effect that is not suppressed by Smo overexpression. In support of this model, Ptc has been shown to interact physically with the adaptor protein DRAL (Fhl2), which in turn recruits one of the caspase recruitment (CARD)-domain containing proteins TUCAN (Card8) or NALP1 (NLR family, pyrin domain containing 1; Nlrp1), as well as caspase 9, thereby activating caspase 9-dependent apoptosis (Brennan et al., 2012).

Perspectives

Hh signalling has emerged as one of the key pathways regulating cell fate specification, differentiation and tissue homeostasis. The list of processes involving Hh continues to grow, as does the body of literature investigating its functions and mechanisms of action. Despite the enormous increase in knowledge about the pathway over the past two decades, there are still many areas where understanding remains incomplete. Major unresolved questions concern how Ptc/Ptch regulates Smo activity, the significance of the dynamic distributions of pathway components in the PC, and which of the various mechanisms proposed to mediate the release and transport of Hh proteins are physiologically important. Future

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biochemical, structural and *in vivo* imaging analyses should help to resolve these puzzles.

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References

Allen, B. L., Song, J. Y., Izzi, L., Althaus, I. W., Kang, J.-S., Charron, F., Krauss, R. S. and McMahon, A. P. (2011). Overlapping roles and collective requirement for the coreceptors GAS1, CDO, and BOC in SHH pathway function. *Dev. Cell* **20**, 775-787.

Beachy, P. A., Hymowitz, S. G., Lazarus, R. A., Leahy, D. J. and Siebold, C. (2010). Interactions between Hedgehog proteins and their binding partners come into view. *Genes Dev.* **24**, 2001-2012.

Bellusci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G. and Hogan, B. L. (1997). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **124**, 53-63.

Brennan, D., Chen, X., Cheng, L., Mahoney, M. and Riobo, N. A. (2012). Noncanonical Hedgehog signaling. *Vitam. Horm.* **88**, 55-72.

Briscoe, J. and Thérond, P. P. (2013). The mechanisms of Hedgehog signalling and its roles in development and disease. *Nat. Rev. Mol. Cell Biol.* **14**, 418-431.

Chen, M.-H., Gao, N., Kawakami, T. and Chuang, P.-T. (2005). Mice deficient in the fused homolog do not exhibit phenotypes indicative of perturbed hedgehog signaling during embryonic development. *Mol. Cell. Biol.* **25**, 7042-7053.

Cheng, S. Y. and Bishop, J. M. (2002). Suppressor of Fused represses Gli-mediated transcription by recruiting the SAP18-mSin3 corepressor complex. *Proc. Natl. Acad. Sci. USA* **99**, 5442-5447.

D'Angelo, G., Matusek, T., Pizette, S. and Thérond, P. P. (2015). Endocytosis of Hedgehog through dispatched regulates long-range signaling. *Dev. Cell* **32**, 290-303.

Dessaud, E., McMahon, A. P. and Briscoe, J. (2008). Pattern formation in the vertebrate neural tube: a sonic hedgehog morphogen-regulated transcriptional network. *Development* **135**, 2489-2503.

Goetz, S. C. and Anderson, K. V. (2010). The primary cilium: a signalling centre during vertebrate development. *Nat. Rev. Genet.* **11**, 331-344.

Gradilla, A.-C., González, E., Seijo, I., Andrés, G., Bischoff, M., González-Méndez, L., Sánchez, V., Callejo, A., Ibáñez, C., Guerra, M. et al. (2014). Exosomes as Hedgehog carriers in cytoneme-mediated transport and secretion. *Nat. Commun.* **5**, 5649.

Hammerschmidt, M., Bitgood, M. J. and McMahon, A. P. (1996). Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. *Genes Dev.* **10**, 647-658.

Hartl, T. A. and Scott, M. P. (2014). Wing tips: the wing disc as a platform for studying Hedgehog signaling. *Methods* **68**, 199-206.

Haycraft, C. J., Banizs, B., Aydin-Son, Y., Zhang, Q., Michaud, E. J. and Yoder, B. K. (2005). Gli2 and Gli3 localize to cilia and require the intraflagellar transport protein polaris for processing and function. *PLoS Genet.* **1**, e53.

He, M., Subramanian, R., Bangs, F., Omelchenko, T., Liem, K. F., Jr, Kapoor, T. M. and Anderson, K. V. (2014). The kinesin-4 protein Kif7 regulates mammalian Hedgehog signalling by organizing the cilium tip compartment. *Nat. Cell Biol.* **16**, 663-672.

Holtz, A. M., Griffiths, S. C., Davis, S. J., Bishop, B., Siebold, C. and Allen, B. L. (2015). Secreted HHIP1 interacts with heparan sulfate and regulates Hedgehog ligand localization and function. *J. Cell Biol.* **209**, 739-758.

Huang, P. and Schier, A. F. (2009). Dampened Hedgehog signaling but normal Wnt signaling in zebrafish without cilia. *Development* **136**, 3089-3098.

Huangfu, D., Liu, A., Rakeman, A. S., Murcia, N. S., Niswander, L. and Anderson, K. V. (2003). Hedgehog signalling in the mouse requires intraflagellar transport proteins. *Nature* **426**, 83-87.

Incardona, J. P., Gruenberg, J. and Roelink, H. (2002). Sonic hedgehog induces the segregation of patched and smoothened in endosomes. *Curr. Biol.* **12**, 983-995.

Ingham, P. W. and McMahon, A. P. (2001). Hedgehog signaling in animal development: paradigms and principles. *Genes Dev.* **15**, 3059-3087.

Ingham, P. W. and McMahon, A. P. (2009). Hedgehog signalling: Kif7 is not that fishy after all. *Curr. Biol.* **19**, R729-R731.

Ingham, P. W. and Placzek, M. (2006). Orchestrating ontogenesis: variations on a theme by sonic hedgehog. *Nat. Rev. Genet.* **7**, 841-850.

Ingham, P. W., Nakano, Y. and Seger, C. (2011). Mechanisms and functions of Hedgehog signalling across the metazoa. *Nat. Rev. Genet.* **12**, 393-406.

Jakobs, P., Exner, S., Schürmann, S., Pickhinke, U., Bandari, S., Ortmann, C., Kupich, S., Schulz, P., Hansen, U., Seidler, D. G. et al. (2014). Scube2 enhances proteolytic Shh processing from the surface of Shh-producing cells. *J. Cell Sci.* **127**, 1726-1737.

Khaliullina, H., Bilgin, M., Sampaio, J. L., Shevchenko, A. and Eaton, S. (2015). Endocannabinoids are conserved inhibitors of the Hedgehog pathway. *Proc. Natl. Acad. Sci. USA* **112**, 3415-3420.

Kornberg, T. B. and Roy, S. (2014). Cytonemes as specialized signaling filopodia. *Development* **141**, 729-736.

Lopez-Rios, J., Duchesne, A., Speziale, D., Andrey, G., Peterson, K. A., Germann, P., Ünal, E., Liu, J., Floriot, S., Barbey, S. et al. (2014). Attenuated sensing of SHH by Ptch1 underlies evolution of bovine limbs. *Nature* **511**, 46-51.

Matusek, T., Wendler, F., Polès, S., Pizette, S., D'Angelo, G., Fürthauer, M. and Thérond, P. P. (2014). The ESCRT machinery regulates the secretion and long-range activity of Hedgehog. *Nature* **516**, 99-103.

Maurya, A. K., Ben, J., Zhao, Z., Lee, R. T. H., Niah, W., Ng, A. S. M., Iyu, A., Yu, W., Elworthy, S., van Eeden, F. J. M. et al. (2013). Positive and negative regulation of Gli activity by Kif7 in the zebrafish embryo. *PLoS Genet.* **9**, e1003955.

McMahon, A. P., Ingham, P. W. and Tabin, C. J. (2003). Developmental roles and clinical significance of hedgehog signaling. *Curr. Top. Dev. Biol.* **53**, 1-114.

Merchant, M., Vajdos, F. F., Ultsch, M., Maun, H. R., Wendt, U., Cannon, J., Desmarais, W., Lazarus, R. A., de Vos, A. M. and de Sauvage, F. J. (2004). Suppressor of fused regulates Gli activity through a dual binding mechanism. *Mol. Cell. Biol.* **24**, 8627-8641.

Milenkovic, L., Weiss, L. E., Yoon, J., Roth, T. L., Su, Y. S., Sahl, S. J., Scott, M. P. and Moerner, W. E. (2015). Single-molecule imaging of Hedgehog pathway protein Smoothened in primary cilia reveals binding events regulated by Patched1. *Proc. Natl. Acad. Sci. USA* **112**, 8320-8325.

Mukhopadhyay, S., Wen, X., Ratti, N., Loktev, A., Rangell, L., Scales, S. J. and Jackson, P. K. (2013). The ciliary G-protein-coupled receptor Gpr161 negatively regulates the sonic Hedgehog pathway via cAMP signaling. *Cell* **152**, 210-223.

Nachtergaele, S., Whalen, D. M., Mydock, L. K., Zhao, Z., Malinauskas, T., Krishnan, K., Ingham, P. W., Covey, D. F., Siebold, C. and Rohatgi, R. (2013). Structure and function of the Smoothened extracellular domain in vertebrate Hedgehog signaling. *eLife* **2**, e01340.

Ogden, S. K., Fei, D. L., Schilling, N. S., Ahmed, Y. F., Hwa, J. and Robbins, D. J. (2008). G protein Galphai functions immediately downstream of Smoothened in Hedgehog signalling. *Nature* **456**, 967-970.

Ortmann, C., Pickhinke, U., Exner, S., Ohlig, S., Lawrence, R., Jboor, H., Dreier, R. and Grobe, K. (2015). Sonic hedgehog processing and release are regulated by glypican heparan sulfate proteoglycans. *J. Cell Sci.* **128**, 2374-2385.

Pan, Y., Wang, C. and Wang, B. (2009). Phosphorylation of Gli2 by protein kinase A is required for Gli2 processing and degradation and the Sonic Hedgehog-regulated mouse development. *Dev. Biol.* **326**, 177-189.

Riobo, N. A., Saucy, B., Dilizio, C. and Manning, D. R. (2006). Activation of heterotrimeric G proteins by Smoothened. *Proc. Natl. Acad. Sci. USA* **103**, 12607-12612.

Rohatgi, R., Milenkovic, L. and Scott, M. P. (2007). Patched1 regulates hedgehog signaling at the primary cilium. *Science* **317**, 372-376.

Sanders, T. A., Llagostera, E. and Barna, M. (2013). Specialized filopodia direct long-range transport of SHH during vertebrate tissue patterning. *Nature* **497**, 628-632.

Shi, Q., Li, S., Jia, J. and Jiang, J. (2011). The Hedgehog-induced Smoothened conformational switch assembles a signaling complex that activates Fused by promoting its dimerization and phosphorylation. *Development* **138**, 4219-4231.

Shin, K., Lee, J., Guo, N., Kim, J., Lim, A., Qu, L., Mysorekar, I. U. and Beachy, P. A. (2011). Hedgehog/Wnt feedback supports regenerative proliferation of epithelial stem cells in bladder. *Nature* **472**, 110-114.

Su, Y., Ospina, J. K., Zhang, J., Michelson, A. P., Schoen, A. M. and Zhu, A. J. (2011). Sequential phosphorylation of smoothened transduces graded hedgehog signaling. *Sci. Signal.* **4**, ra43.

Svärd, J., Heby-Henricson, K., Henricson, K. H., Persson-Lek, M., Rozell, B., Lauth, M., Bergström, A., Ericson, J., Toftgård, R. and Teglund, S. (2006). Genetic elimination of Suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. *Dev. Cell* **10**, 187-197.

Teperino, R., Amann, S., Bayer, M., McGee, S. L., Loipetzberger, A., Connor, T., Jaeger, C., Kammerer, B., Winter, L., Wiche, G. et al. (2012). Hedgehog partial agonism drives Warburg-like metabolism in muscle and brown fat. *Cell* **151**, 414-426.

Tukachinsky, H., Lopez, L. V. and Salic, A. (2010). A mechanism for vertebrate Hedgehog signaling: recruitment to cilia and dissociation of SuFu-Gli protein complexes. *J. Cell Biol.* **191**, 415-428.

Wang, G. and Jiang, J. (2004). Multiple Cos2/Ci interactions regulate Ci subcellular localization through microtubule dependent and independent mechanisms. *Dev. Biol.* **268**, 493-505.

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Wang, J., Cao, J., Dickson, A. L. and Poss, K. D. (2015). Epicardial regeneration is guided by cardiac outflow tract and Hedgehog signalling. *Nature* **522**, 226-230.

Wehner, D. and Weidinger, G. (2015). Signaling networks organizing regenerative growth of the zebrafish fin. *Trends Genet.* **31**, 336-343.

Yam, P. T. and Charron, F. (2013). Signaling mechanisms of non-conventional axon guidance cues: the Shh, BMP and Wnt morphogens. *Curr. Opin. Neurobiol.* **23**, 965-973.

Yam, P. T., Langlois, S. D., Morin, S. and Charron, F. (2009). Sonic Hedgehog guides axons through a noncanonical, Src-family-kinase-dependent signaling pathway. *Neuron* **62**, 349-362.

Yavari, A., Nagaraj, R., Owusu-Ansah, E., Folick, A., Ngo, K., Hillman, T., Call, G., Rohatgi, R., Scott, M. P. and Banerjee, U. (2010). Role of lipid metabolism in smoothened derepression in hedgehog signaling. *Dev. Cell* **19**, 54-65.

Ye, F., Breslow, D. K., Koslover, E. F., Spakowitz, A. J., Nelson, W. J. and Nachury, M. V. (2013). Single molecule imaging reveals a major role for diffusion in the exploration of ciliary space by signaling receptors. *eLife* **2**, e00654.

Yin, Y., Bangs, F., Paton, I. R., Prescott, A., James, J., Davey, M. G., Whitley, P., Genikhovich, G., Technau, U., Burt, D. W. et al. (2009). The Talpid3 gene (KIAA0586) encodes a centrosomal protein that is essential for primary cilia formation. *Development* **136**, 655-664.

Zhang, W., Zhao, Y., Tong, C., Wang, G., Wang, B., Jia, J. and Jiang, J. (2005). Hedgehog-regulated Costal2-kinase complexes control phosphorylation and proteolytic processing of Cubitus interruptus. *Dev. Cell* **8**, 267-278.

Zhou, Q. and Kalderon, D. (2011). Hedgehog activates fused through phosphorylation to elicit a full spectrum of pathway responses. *Dev. Cell* **20**, 802-814.