Protein family review

# The Hedgehog protein family

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Published: 19 November 2008

*Genome **Biology*** 2008, **9:**241 (doi:10.1186/gb-2008-9-11-241)

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2008/9/11/241



## Summary

The Hedgehog (Hh) pathway is one of the fundamental signal transduction pathways in animal development and is also involved in stem-cell maintenance and carcinogenesis. The *hedgehog* (*hh*) gene was first discovered in *Drosophila*, and members of the family have since been found in most metazoa. Hh proteins are composed of two domains, an amino-terminal domain HhN, which has the biological signal activity, and a carboxy-terminal autocatalytic domain HhC, which cleaves Hh into two parts in an intramolecular reaction and adds a cholesterol moiety to HhN. HhC has sequence similarity to the self-splicing inteins, and the shared region is termed Hint. New classes of proteins containing the Hint domain have been discovered recently in bacteria and eukaryotes, and the Hog class, of which Hh proteins comprise one family, is widespread throughout eukaryotes. The non-Hh Hog proteins have carboxy-terminal domains (the Hog domain) highly similar to HhC, although they lack the HhN domain, and instead have other amino-terminal domains. Hog proteins are found in many protists, but the Hh family emerged only in early metazoan evolution. HhN is modified by cholesterol at its carboxyl terminus and by palmitate at its amino terminus in both flies and mammals. The modified HhN is released from the cell and travels through the extracellular space. On binding its receptor Patched, it relieves the inhibition that Patched exerts on Smoothened, a G-protein-coupled receptor. The resulting signaling cascade converges on the transcription factor Cubitus interruptus (Ci), or its mammalian counterparts, the Gli proteins, which activate or repress target genes.

## Gene organization and evolutionary history

Hedgehog (Hh) proteins are composed of two distinct domains, the amino-terminal 'Hedge' domain (HhN), and the carboxy-terminal 'Hog' domain (HhC) (Figure 1 and Box 1). The founding member of the *hh* gene family was first discovered in genetic screens in *Drosophila melanogaster* [1] and, once the gene was cloned [2-4], vertebrate members were soon found [5-7]. *Drosophila* has a single *hh* gene, mammals have three paralogous genes, called Sonic Hedgehog (*Shh*), Indian Hedgehog (*Ihh*), and Desert Hedgehog (*Dhh*), and the cnidarian *Nematostella vectensis* has two paralogous *hh* genes, *Nv_HH1* and *Nv_HH2* [8]. The *hh* gene family is present throughout the Eumetazoa, although it has been lost in some nematodes. For example, *Caenorhabditis elegans* has no *hh* gene but has other genes related to *hh* via the Hog domain. These *hh*-related genes have been grouped into different families, such as Warthog (*wrt*), Groundhog (*grd*), and Quahog (*qua*), and are characterized by having amino-terminal sequences distinct from HhN [9,10].

Soon after the discovery of the fly and vertebrate Hh proteins, it was noticed that their carboxy-terminal autoproteolytic domains were similar in sequence to the self-splicing inteins [11]. Inteins are protein sequences that autocatalytically splice themselves out of longer protein precursors - analogous to introns - and ligate the flanking regions into a functional protein [12,13]. The determination of the X-ray structure of the *Drosophila* HhC domain confirmed this similarity, and the region of similarity was named the Hint module [14] (see Figure 1). More recently, new classes of Hint-containing proteins with various types of processing activity have been recognized in bacteria and eukaryotes [10,13,15,16] (Figure 2). Intein-containing genes

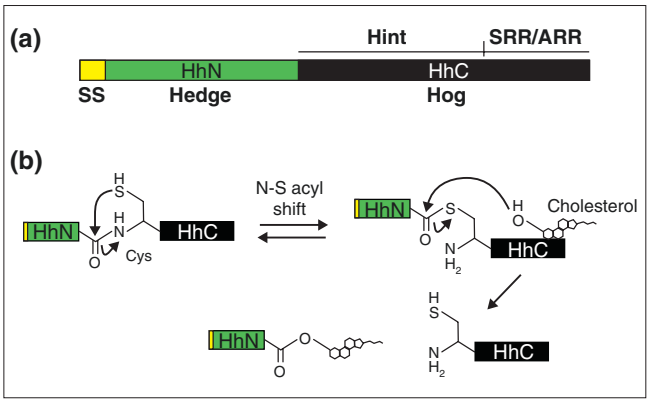


**Figure 1**
Structural features of Hh proteins. **(a)** Signal peptide sequence for protein export (SS, yellow), the amino-terminal signaling domain (HhN, green), and the autocatalytic carboxy-terminal domain (HhC, black) are indicated. Both HhN and HhC domains are also found in proteins other than the Hh family, and are therefore globally referred to as 'Hedge' and 'Hog', respectively. The Hog domain itself can be separated into two regions; the first two-thirds shares similarity with self-splicing inteins, and this module has been named Hint, whereas the carboxy-terminal third binds cholesterol in Hh proteins and has been named the sterol-recognition region (SRR) [14]. In Hog proteins other than Hh, that is, Hh-related proteins, this region is referred to as ARR (adduct recognition region) [21], as the nature of the adduct is not known. **(b)** Intramolecular autoprocessing of Hh. Acids and bases assisting in catalysis are not shown (figure adapted from [14,70]).

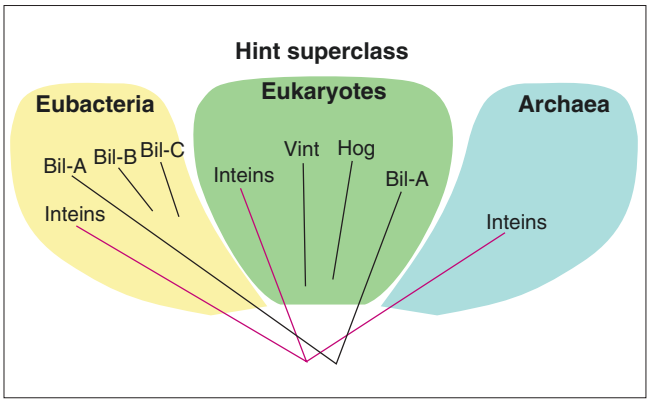


**Figure 2**
Distribution of Hint superclass genes in the three domains of life. Hint genes can be divided into several different classes: inteins; Bil-A (bacterial intein-like genes type A); Bil-B; a new class referred to here as Bil-C [10,13]; Vint (VWA domain and Hint domain proteins) [10]; and Hog.

are present in all three kingdoms of life, but Hog genes and Vint genes - a novel class of proteins sharing a VWA domain (von Willebrand factor type A domain) and a Hint domain - are known only from eukaryotes at present (Figure 2). Initially, Hog genes, primarily members of the Hh family, were found only in metazoa, but they have recently been found in many different branches of protists [10,13,17,18] (Figure 3). This widespread distribution indicates that the Hog domain must be of ancient origin and have emerged early in eukaryote evolution. Hog genes are absent in higher plants and several fungal clades, which is presumably due to

**Box 1. Terminology**

**Hint domain/module:** an autoproteolytic domain/module originally described in Hedgehog proteins and self-splicing inteins. The Hint-containing group of proteins encompasses several distinct classes, such as inteins, the Hog proteins (including the Hh family), as well as as Bil-A, Bil-B, and Vint.

**Hog proteins:** class of Hint proteins with a distinct subtype of Hint domain and a carboxy-terminal ARR found in many eukaryotic phyla. The Hint and ARR regions together comprise the Hog domain.

**Hedgehog (Hh):** one family of Hog proteins found in eumetazoa, composed of an amino-terminal Hedge (HhN) domain and a carboxy-terminal Hog (HhC) domain.

**Hedge domain:** comprehensive term for the amino-terminal domain of Hh proteins and of Hedgling proteins (which lack a Hog domain).

**HhN** and **HhC:** amino-terminal and carboxy-terminal domains specifically of Hh family proteins.

**Hh-related genes:** a comprehensive term used for those Hog proteins that have amino-terminal domains different from that of Hh, for example, the Quahog, Warthog, and Groundhog families in nematodes.

**SRR:** sterol-recognition region, the cholesterol-binding site of HhC.

**ARR:** adduct recognition region in the Hog domain of Hh-related proteins

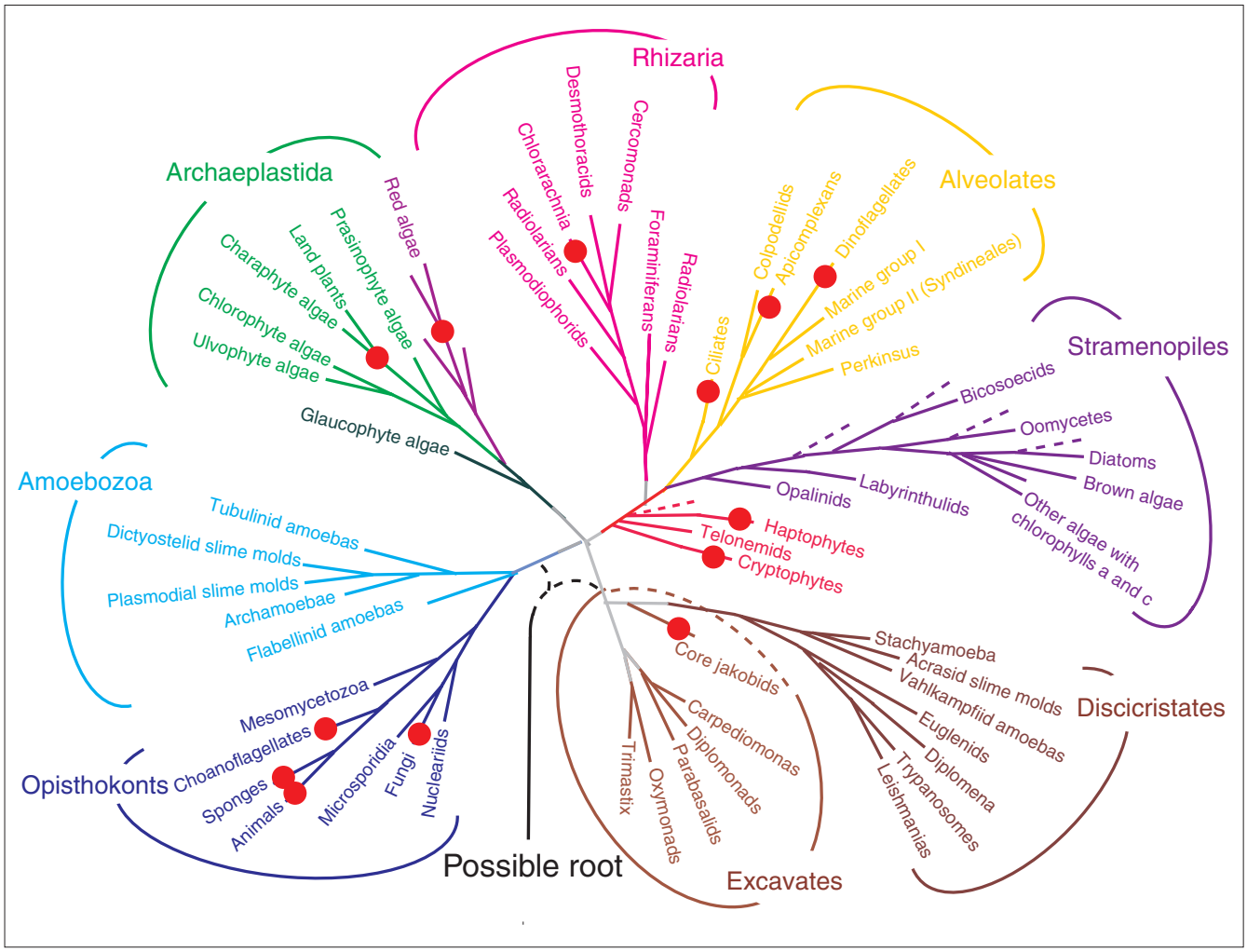


**Figure 3**
Consensus phylogenetic tree of eukaryotes. The branches where Hog domain containing proteins are found are indicated with red dots. With permission from Sandra Baldauf, (see, also [71]).

gene loss. Many of the protist Hog proteins, as well as the metazoan non-Hh Hog proteins - referred to as Hh-related proteins - have putative secreted domains upstream of the Hog domain [10]. In most cases these upstream regions show conservation only with related Hog genes within the same phylum, suggesting a gradual evolution of the amino-terminal regions within each phylum. In a few instances, such as the fungus *Glomus mosseae* [17], the choanoflagellate *Monosiga ovata* [18], and the sponge *Amphimedon queenslandica* [19], the Hog domain is fused to other well-conserved domains, indicative of a merging of two distinct domains.

The Hedge domain seems to be of more recent origin. It has been found in sponges and Cnidaria in a large extracellular membrane protein called Hedgling [19]. In addition to the Hedge domain at the amino terminus, Hedgling contains many additional domains, such as a VWA domain and numerous cadherin repeats, but lacks a Hog domain [10,19]. A second, divergent fragment of a Hedge domain has been found in the sponge *Oscarella carmela* that also seems to lack a Hog domain [10,20]. At present, no *hh* genes have been found in sponges, but they are present in Cnidaria. Two scenarios can be envisaged for the emergence of Hh proteins proper (Figure 4). One is that the Hedge domain evolved from a secreted amino-terminal domain already associated with the Hog domain. Hedgling is then derived from Hh by a 'split' of Hedge from Hog before the emergence of sponges. The other is that the Hedge domain evolved in an extracellular protein such as Hedgling. During the emergence of Eumetazoa, the Hedge domain 'fused' with a Hog protein to give rise to Hh. Examples of both domain split and loss and domain-merging events are documented for Hog proteins, and therefore do not help to discriminate between alternative scenarios.

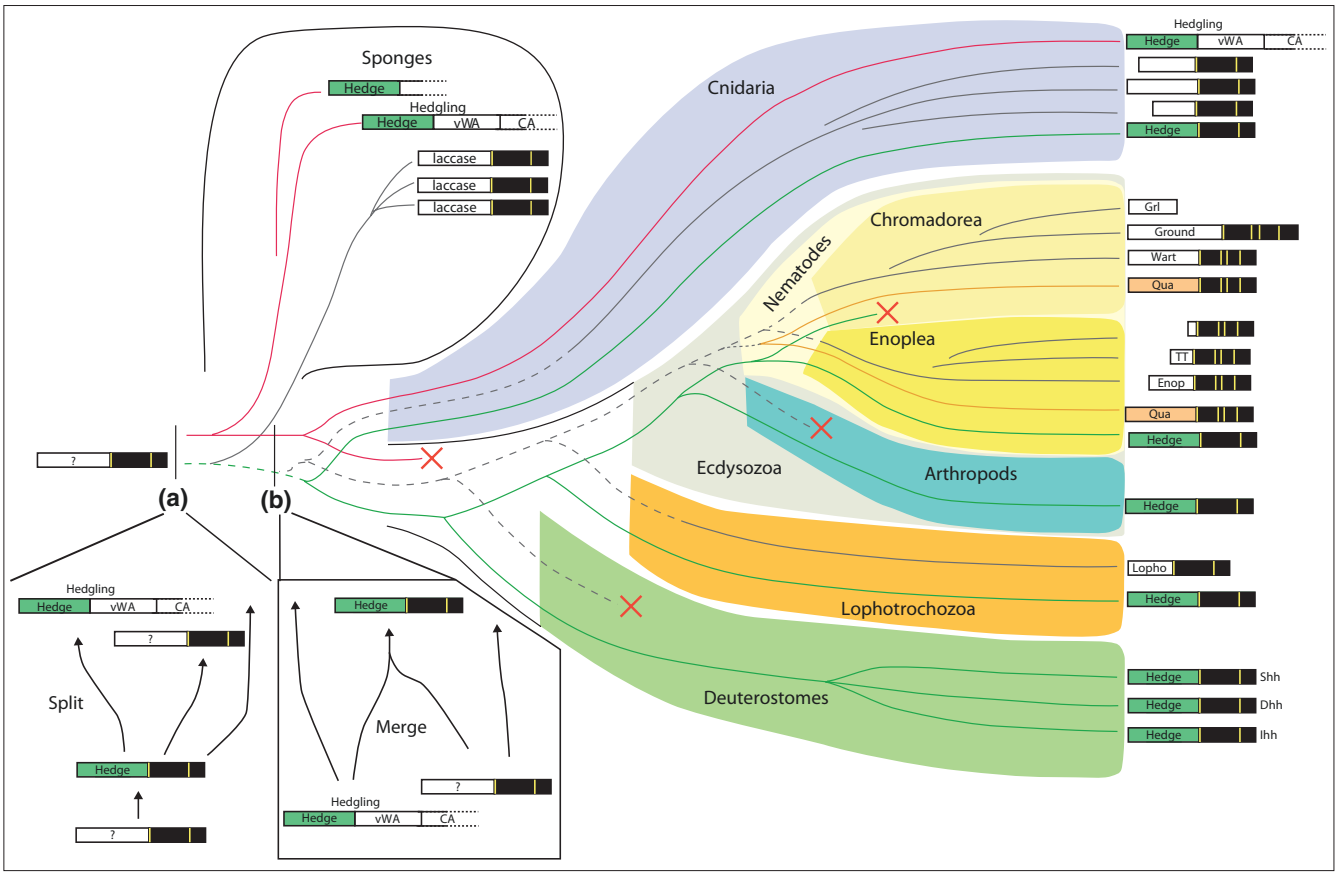


**Figure 4**
One possible scenario for the evolution of *hh* and *hh*-related genes in metazoa. Different phylogenetic branches are outlined, and gene families known at present are shown. Dotted lines indicate uncertain evolutionary connections. Hedgling genes are currently known only from sponges and Cnidaria [8,10,19]. The Hh family could have originated in two possible ways. **(a)** The Hedge domain evolved concomitantly with the Hog domain from a protist Hog protein before the emergence of the Metazoa. A duplication of the Hedge domain and merger with an extracellular protein gave rise to the Hedgling gene. **(b)** No *hh* gene existed at the emergence of sponges. The Hedge domain of a Hedgling gene duplicated and merged with a Hog gene to give rise to *hh* in early Eumetazoa. Cnidaria, Lophotrochozoa and nematodes contain both Hh as well as other Hog family genes. The phylogenetic analysis cannot unequivocally resolve whether these other families originated from a single ancestor in Eumetazoa - as shown here with dotted lines - or whether, at least in some phyla, duplication and divergence from a *hh* gene gave rise to new families in particular phyla.

Very recent findings have led to a revised understanding of the evolution of *hh* genes and the *hh*-related genes in metazoa. In *Drosophila* and vertebrates only *hh* genes are found, but both *hh* and *hh*-related genes are present in the Cnidaria, nematodes and also the Lophotrochozoa [8,10]. I have searched the genome sequences of two lophotrochozoan species, the limpet *Lottia gigantea* and the polychaete worm *Capitella* I ECS-2004, and retrieved one *hh* gene and six *hh*-related genes from *L. gigantea* and one *hh* gene and one *hh*-related gene from *Capitella*. These sequences have been combined with previously published sequences to generate a new phylogenetic tree based on the Hog domain (Figure 5). The most interesting observation from the tree is that the *hh*-related genes Cap_213608 and Lg_236513 form a clade, and these two sequences also share sequence similarity just upstream of the Hog domain. Therefore, it seems likely that a new *hh*-related gene family, which I refer to as 'Lophohog', exists in the Lophotrochozoa and developed in parallel with Hh. On the basis of this observation, the following model could be proposed for the evolution of *hh* and *hh*-related genes in metazoa (see Figure 4). I suggest that at least one *hh* and one *hh*-related gene existed at the origin of the Eumetazoa, giving rise to the *hh* and *hh*-related genes in the Cnidaria, the Lophotrochozoa, and nematodes. In *Drosophila* and deuterostomes the *hh*-related gene was lost, whereas in the nematode branch leading to *C. elegans*, *hh* was lost. The most radical alternative scenario would be that the *hh*-related genes in Cnidaria, Lophotrochozoa, and nematodes are all derived independently from a *hh* gene in each phylum. Intermediate scenarios, where *hh*-related genes evolved from a *hh* gene only in one or two phyla, could also be possible. Phylogenetic analysis does not give definitive answers yet, but may resolve the question in the future, when additional genomes are sequenced.

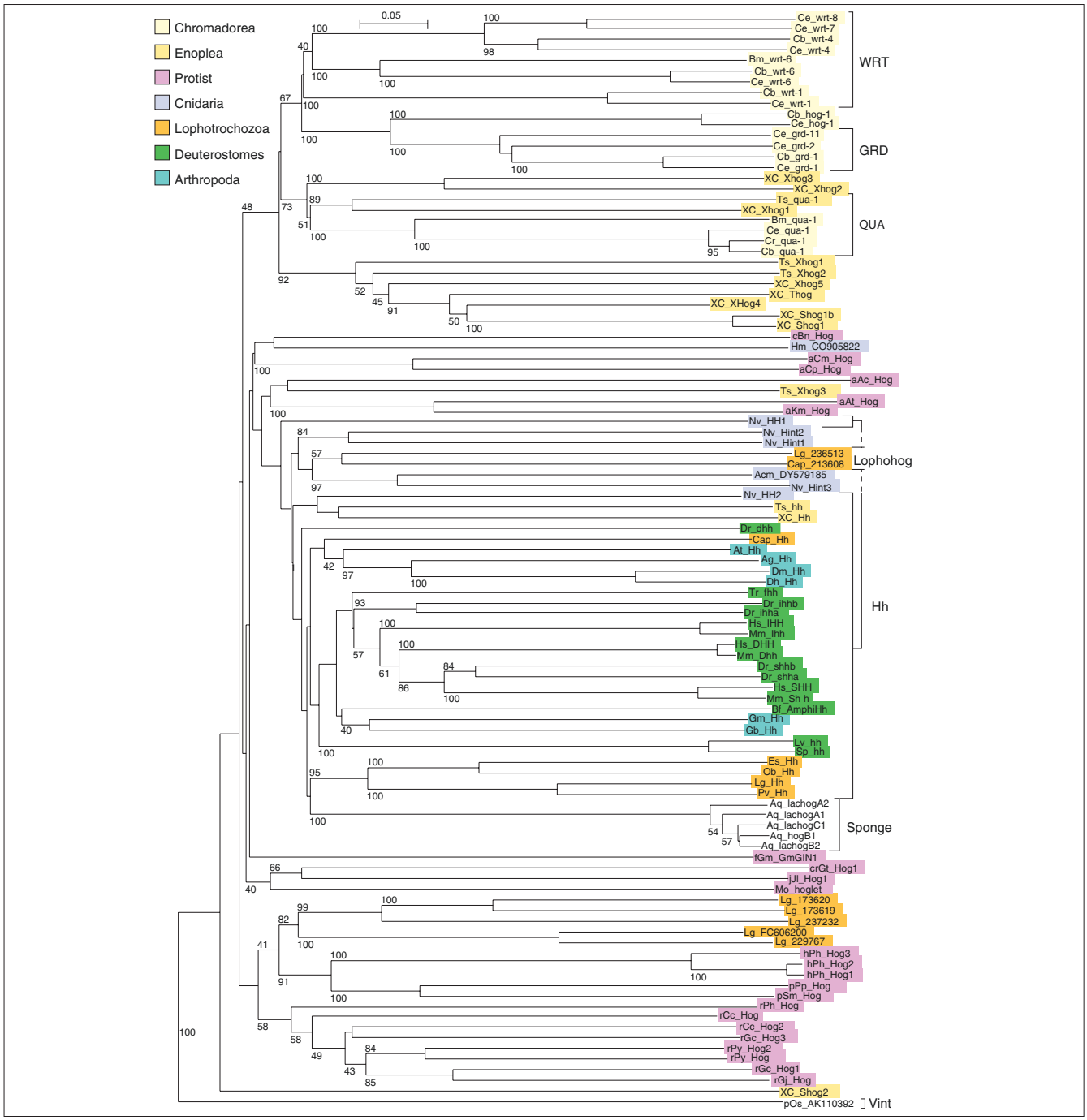


**Figure 5**

Neighbor-joining phylogenetic tree of eukaryote Hog domain protein sequences. The Hh, Groundhog (Grd), Warthog (Wrt), Quahog (Qua), and new Lophohog families are indicated. Sequence names are color-coded according to phyletic divisions, except for sponges. Chromadorea and Enoplea are two major nematode divisions. Protist is loosely used to encompass all non-metazoans. The Hint domains of Vint proteins were used as outgroup and bootstrap values ≥ 40 % are shown. Most of the sequences and the analysis methods are described in [10]. Additional sequences were added to this analysis from sponges [19], and BLAST searches were carried out at JGI [72] of the genomes of *L. gigantea* and *Capitella* I ECS-2004. From *Capitella* I ECS-2004 one *hh* and one *hh*-related gene were retrieved, and from *L. gigantea* one *hh* and six other Hog genes were retrieved. *Capitella* Cap_213608 and *L. gigantea* Lg_236513, which encodes an export signal peptide, form a clade, although not with high bootstrap significance. Interestingly, this clade clusters with the Cnidarian *hh*-related genes - although bootstrap values are insignificant. Five *L. gigantea* Hog genes (Lg_173620, Lg_173619, Lg_237232, Lg_FC606200, Lg_229767) form a distinct clade, but these genes are very divergent from the Hog domains of the other metazoan genes. These genes encode only a few residues upstream of the Hog domain (7-15), and lack an export signal peptide. This unusual structure is confirmed by multiple expressed sequence tags (ESTs) for each gene. Do these genes represent a highly divergent form of Hog-only proteins in this gastropod, or do they stem from another organism, perhaps some ciliated protozoan parasite found in *L. gigantea* [73]? More analysis will be necessary to resolve this.

## Characteristic structural features

Hh proteins are synthesized as precursor proteins (about 400-460 amino acids long) and comprise several different motifs and domains: a signal peptide for protein export, a secreted amino-terminal HhN (Hedge) domain that acts as a signaling molecule, and an autocatalytic carboxy-terminal HhC (Hog) domain that contains a Hint module (see Figure 1). Multiple sequence alignments of the HhN and HhC domains defining the conserved residues and features have been presented in [10]. HhC binds cholesterol in the sterol-recognition region (SRR) [21]. The catalytic activity of the Hint module cleaves Hh into two parts and adds the cholesterol moiety to the carboxyl terminus of HhN (Figure 1b). The structure of *Drosophila* HhC has been determined using X-ray crystallography and shows a high congruence with that of inteins [14]. The structure is globular, composed of β strands, and starts with a cysteine residue critical for autoprocessing (Figure 1b). The nematode Hh-related protein WRT-1 was shown to be autoprocessed like Hh [22]. Given that the critical residues of the active site of HhC are well conserved among Hog proteins [10,14], it can be assumed that most, if not all, are autoprocessed. However, it is not known what adduct binds to the adduct-recognition region (ARR) of Hh-related proteins. Intriguingly, the ARR regions of some of the protist Hog proteins contain motifs conserved with the Hh SRR [10], suggesting that sterol binding might be an ancient feature.

The structure of the HhN domain of mouse Shh has also been determined [23]. It is a relatively globular domain with two antiparallel α helices and several β strands wrapping one face of the two helixes. Although it was found to have a potential catalytic site, no enzymatic activity has been uncovered so far [24]. In addition to the cholesterol modification, the HhN domain is also modified at its amino terminus by palmitate through the action of a transmembrane acyltransferase, named Skinny hedgehog (Ski, also known as Rasp) in *Drosophila* [25], and hedgehog acyltransferase (HHAT) in mammals [26]. Because of these lipid modifications, the modified HhN domain (M-HhN) can form multimeric complexes [27,28] and can interact with lipoproteins [29]. *Drosophila* Ihog (interference hedgehog) and its mammalian orthologs Cdo and Boc are M-HhN-interacting proteins that are required for normal Hh signaling. They are type I integral membrane proteins with four extracellular immunoglobulin-like domains and two extracellular fibronectin type III domains. Biochemical and structural studies of complexes of *Drosophila* HhN and Ihog show that heparin induces dimerization of Ihog, a prerequisite for high-affinity interactions between M-HhN and Ihog [30]. Biochemical and structural studies of complexes of mouse ShhN and Cdo revealed a different mode of binding, where a calcium-binding site in ShhN is important for the interaction [31]. Therefore, although the structures of fly HhN and mouse ShhN are conserved, the mode of interaction is not necessarily conserved in evolution.

## Localization and function

An export signal peptide targets newly synthesized Hh to the endoplasmic reticulum, where autoprocessing, as well as palmitoylation, of the HhN domain occurs [26,28]. The modified HhN is released from the cell with the aid of the 12-pass transmembrane protein Dispatched (Disp). Once released into the extracellular environment, M-HhN interacts with a number of different proteins: the heparan-sulfate proteoglycan Dally-like (Dlp), and the proteins Ihog and growth-arrest-specific 1 (Gas1) are positive regulators of Hh signaling, whereas Hh-interacting protein (Hip) acts as a negative regulator by sequestering M-HhN. The lipid modification of HhN as well as the extracellular protein interactions influence its extracellular movement and ensure correct short- and long-range signaling (see, for example, [28]).

The key function of M-HhN as an extracellular signal is to inhibit the activity of the receptor Patched (Ptc), a 12-pass transmembrane protein. Ptc is closely related to Disp and shares similarity with the bacterial family of resistance-nodulation division (RND) proton pumps that transport small molecules across membranes. Numerous reviews deal with the biological function of the Hh pathway and its components [32-52]. Figure 6 shows a summary of the pathway composed from *Drosophila* and mammalian data (although a number of important differences exist between the pathways in these two groups of organisms). Briefly, in the absence of M-HhN binding, Ptc represses a signaling pathway that acts through Smoothened (Smo), a seven-pass G-protein-coupled receptor. Smo is negatively regulated by pro-vitamin D3, and is positively, but indirectly, regulated by oxysterols (oxygenated derivatives of cholesterol) [53-55]. 7-Dehydrocholesterol reductase, which converts pro-vitamin D3 into cholesterol, is also a regulator of Hh signaling [56]. Another important aspect of Smo activity is its subcellular localization. When M-HhN binds to Ptc, the complex is internalized while Smo translocates to the cell membrane or - in mammals - to the primary cilia. Localization of Smo to the primary cilia is a fundamental requirement for the pathway to be active, and in the absence of M-HhN, Ptc inhibits this localization [57]. How exactly Ptc inhibits Smo is still not clear and numerous models are being contemplated (see, for example, [38,41,52]). Because of the similarity of Ptc to bacterial transporters, Ptc could secrete a pro-vitamin D3 or related molecule to inhibit Smo. Activated Smo is phosphorylated and signals via a cascade of microtubule-associated proteins to the nucleus, where the transcription factor Cubitus interruptus (Ci) in *Drosophila* or its mammalian counterparts, the Gli transcription factors, activate or repress target genes. Among the many target genes regulated by mammalian Gli1 are those for Ptc and Gli1 themselves. This results in feedback loops in which upregulation of Ptc leads to negative feedback, whereas upregulation of Gli1 leads to positive feedback.

In animal development, the secreted M-HhN moiety functions as a morphogen. The Hh signaling pathway plays

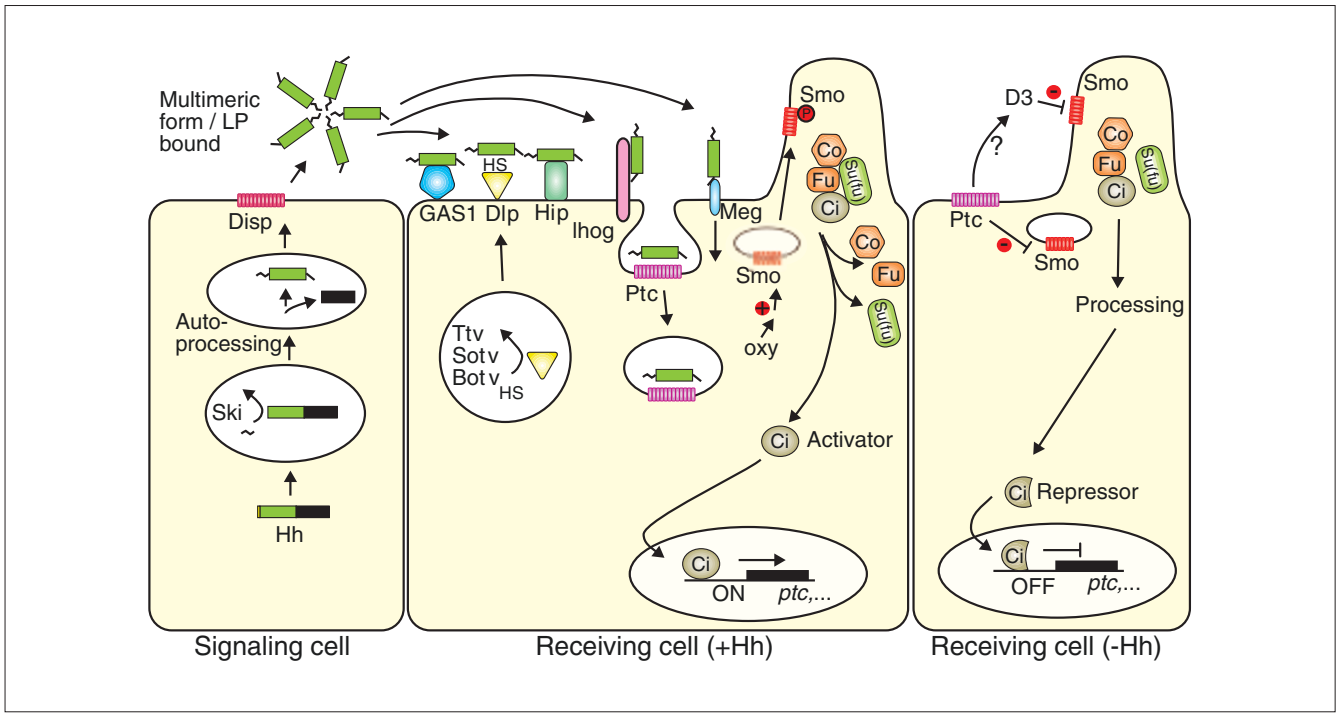


**Figure 6**
A simplified Hh signaling pathway, constructed from combined *Drosophila* and mammalian data. Hh is targeted to the endoplasmic reticulum by its signal peptide, is palmitoylated at its amino terminus by Rasp/Skinny Hedgehog (Ski), and autoprocessed. Lipidated HhN (M-HhN) is released by Dispatched (Disp) and forms multimers or associates with lipoproteins (LP) in the extracellular environment [32]. A number of molecules can interact with M-HhN and propagate or modulate its trafficking: the Dally-like protein (Dlp), which is modified by the heparan sulfate (HS) polymerases Tout-velu (Ttv), Sister of tout-velu (Sotv), and Brother of tout-velu (Botv), all members of the EXT family; the Hedgehog-interacting protein (Hip); and the Growth-arrest-specific 1 (Gas1) protein. Hip and Gas1 are not present in *Drosophila*. Megalin (Meg) is most probably involved in the recycling of M-HhN. Ihog is thought to function as co-receptor for M-HhN. M-HhN acts as an antagonistic ligand that represses the function of the receptor Patched (Ptc), a 12-transmembrane protein related to Disp. Binding of M-HhN to Ptc results in internalization. Smoothened (Smo) is a seven-pass membrane receptor, which is key for the transmission of the signal to the nucleus in the Hh pathway. Smo is inhibited by Ptc when not bound by M-HhN. When the inhibitory function of Ptc is released by M-HhN, Smo can translocate to the plasma membrane or - in mammals - to the primary cilium, and active Smo is phosphorylated (red P). Ptc may secrete pro-vitamin D3 or related compounds (D3) to inhibit Smo. Conversely, oxysterols (Oxy) can indirectly activate Smo [52,55]. The Hh pathway downstream of Smo displays some important differences between *Drosophila* and mammals. In *Drosophila*, when Smo is active, the signal passes through a complex comprising the kinesin-like molecule Costal 2 (Cos2), Fused (Fu), Suppressor of fused (Su(fu)) and Cubitus interruptus (Ci), leading to the release of Ci, which can then enter the nucleus to activate transcription. When Smo is inhibited, the Cos2/Fu/Su(fu)/Ci complex remains associated with microtubules, Ci is phosphorylated and is cleaved by Cos2. The Ci fragment now acts as a transcriptional repressor. In mammals, the targeting of Smo to primary cilia is essential for signal transduction. No obvious equivalents of Cos2 and Fu exist in mammals. Instead, Su(fu) has a more prominent role in inhibiting the pathway. Gli1, Gli2, and Gli3 are the mammalian homologs of Ci; Gli1 and Gli2 activate transcription when Smo is active, whereas Gli3 is processed and becomes a repressor when Smo is inhibited. A number of components in the pathway, in particular downstream of Smo, are not shown in this figure.

many important roles in development, including conferring segment polarity on the body segments and patterning the wing in *Drosophila*, and patterning the neural tube in mammals [39,48,58]. Hh is also required for stem-cell maintenance, and mutations in the pathway lead to cancer. Increased activity of the pathway causes basal cell carcinoma and medulloblastoma [37,59-63]. For example, insufficient Ptc function leads to Gorlin syndrome in humans, one feature of which is an increased risk of basal cell skin cancer. In mammals, Shh, Dhh, and Ihh have partially redundant functions. Shh is the most widely expressed of the three paralogs, and regulates development from embryo to adult. Key roles are in patterning the neural tube: Shh is first expressed in the notochord, and later in the floor plate of the neural tube, where it produces a gradient of activity in the ventral neural tube. Shh is also expressed in the zone of polarizing activity of the limb buds and is important for limb and digit formation. Other roles of Shh include inner ear, eye, taste bud, and hair follicle development. Ihh is expressed in the primitive endoderm and is required for bone growth and pancreas development. Shh and Ihh both play roles in cardiovascular development. Dhh is expressed in the gonads, and Dhh-mutant males are sterile [39,48,64].

## Frontiers

Despite substantial insights into the Hh signaling pathway, there are still many gaps in our understanding. How, and in

which forms, the M-HhN morphogen travels from the signaling cells to the target cells requires further investigation. Obviously, the number of potential interactors in the extracellular matrix and extracellular space is vast, and any changes therein could influence how M-HhN propagates. And could the M-HhN domain potentially have functions other than to regulate the Ptc-Smo interaction? Clearly, the amino-terminal domains of Hh-related proteins in protists and nematodes, as well as Hh in Enoplea [10] must have other functions, as there is no *bona fide* Hh signaling pathway in these organisms. The inhibition of Smo by Ptc and the role of sterol compounds also need further investigation to unravel the action of sterols on Smo, and to determine how Ptc is involved in this regulation. The Hh signaling pathway has been compared to the Wnt pathway, another key signaling pathway in development, since some of the molecules in the pathways have similarities to each other [65]. However, the Hh signaling pathway is unusual and different from other signaling pathways in that the primary morphogen, M-HhN, does not directly act on the key receptor, Smo. Perhaps the Smo signaling pathway was originally part of a sterol homeostasis pathway. M-HhN and Ptc could then be viewed as secondary modifiers of the Smo pathway. Did they originally have other functions? For example, the Ptc homolog PTC-1 in *C. elegans* functions in the absence of Smo and plays a role in oocyte cytokinesis [66].

A substantial number of components of the Smo signaling cascade leading to the nucleus have been uncovered, though many of the interactions still need to be better understood. Recently, however, a new Smo response pathway was uncovered that does not depend on transcription activation through Smo [67], opening the possibility that yet other aspects of the pathway downstream of Smo remain to be discovered. The importance of oxysterols in Hh signaling connects the Hh pathway with cholesterol homeostasis [49,52,68,69]. Hence, it will be a formidable challenge to unravel the interactions between sterol compounds, Hh, Ptc and Smo and to comprehend the kinetics and biophysical aspects of their subcellular localization. Understanding of all the regulatory controls and feedback loops in this signaling pathway will ultimately require computational modeling.

## Acknowledgements

I would like to thank Peter Zaphiropoulos for critical reading of the manuscript. TRB is supported by the Center of Biosciences.

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