Novel Mechanisms and Functions for Hedgehog Signaling in Development and Disease
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Citation for published version (APA):

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Part I.

Novel Mechanisms for Hedgehog Signaling
Chapter I.

Hedgehog: an Introduction to an Unusual Signal Transducer

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Adapted from:
Bioessays (2004)

Summary
Hedgehog (Hh) proteins are of pivotal importance for development and maintenance of tissue patterns in adult organisms. Despite the role of Hh proteins in differentiation and tumorigenesis, signal transduction of Hh remains a relatively uncharted area of signaling biochemistry. For proper Hh distribution into tissues, two highly unusual covalent modifications are necessary, palmitoylation of a secreted protein and the attachment of a cholesterol group, making Hh the only established sterolated protein in the animal kingdom. Hh exerts its function via two membrane-bound receptors, Patched and Smoothened; Patched transports (pro-)vitamin D out of cells which inhibits Smoothened. Binding of Hh to Patched impedes this pump function and thus Smoothened inhibition, leading to expression of genetic Hh targets via relief of transcriptional repression. These atypical features make Hh physiology unique in biology and may explain why this field has attracted such significant attention.

Introduction
One of most important concepts to arise from contemporary developmental biology is the realization that in major patterning events throughout the animal kingdom the extrinsic information guiding morphogenesis is provided by a limited number of morphogens. Concentration gradients of these morphogens provide the differentiating cell spatial information to couple cellular position to its appropriate developmental fate. The most important of these morphogens are the fibroblast growth factor (FGF), transforming growth factor (TGF), including the bone morphogenetic proteins and activins, Wingless/Int-1 (WNT), and Hedgehog (Hh) families (Hogan, 1999). The molecular details with respect to signaling of these proteins are arguably least well understood with respect to Hh proteins.

As is to be expected from a morphogen family, a plethora of processes in different classes of the animal kingdom have been described in which members of the Hh protein family are involved. Very often these functions concern embryonic development, most notably the segmental patterning in the fruit fly embryo or digit patterning in the chick limb bud as well as left-right asymmetry of vertebrate embryos. Hh, however, is also vital for the maintenance of tissue patterns in adults as well as tissue salvage following ischemia-reperfusion. Deregulated Hh signaling in adults is implicated in tumorigenesis. As a consequence of this wide range of functions, ever since its discovery in the 1980s, the Hh signaling pathway has attracted substantial research interest and probably will continue to do so as technical advances allow further insight into the molecular details of its signaling. In the present essay we shall attempt to provide the uninitiated reader with some first insights into the features and functions of Hh proteins in both normal and pathological growth control.

The Hh Mutant in Drosophila melanogaster
Most of the components of morphogenetic signaling pathways have been detected by phenotypes visible in mutant Drosophila larvae. The Hh pathway is no exception. In wild-type Drosophila melanogaster larvae, a clear segmented pattern is visible. A band of denticles runs across the anterior half of each segment, whereas the posterior half is smooth (the so-called naked cuticle). In screening for mutations that affect the segmental pattern of Drosophila larvae, Nüsslein-Volhard and Wieschaus discovered a group of mutants that affected patterning within the segments but at the same time left the number of segments unaltered (Nüsslein-Volhard and Wieschaus, 1980). One of these so-called segment polarity
mutants caused denticles to occur not only on the anterior, but also on the posterior half of the segments, covering the back of the larvae with a continuous lane of bristles as shown in Fig. 1B (wild type Fig. 1A). This mutant was therefore named after the spiny little mammal hedgehog.

Although fervently sought after, the cloning of the Hh gene was achieved only in 1992, and a prediction of the protein’s properties was made according to the calculated hydrophobicity of the spliced mRNA (Mohler and Vani, 1992). Originally, the Hh protein was thought to be a transmembrane, non-diffusible signal for neighbouring cells. However, with the exact amino acid sequence of the protein known a year later, it became clear that the hydrophobic region is part of a signal sequence, and Hh was thus proposed to constitute a secretory protein, a notion that turned out to be correct shortly after (Tashiro et al., 1993). At that time, however, one was still unaware of the unusual posttranslational protein modifications and of the autocatalytic properties of Hh necessary for its biological activity (see below).

**HEDGEHOG PROTEIN PROCESSING**

Among the most unusual features of Hh proteins is their unconventional posttranslational processing. Two years after the cloning of the Hh gene, it became apparent that functional Hh is not a full-length product of the mature mRNA (Lee et al., 1994). In order for Hh to exert its biological activity multiple modifications take place and these are summarized in Fig. 2. In addition to removal of the signal sequence, the approximately 45 kD large Hh protein undergoes autocatalytic cleavage (Lee et al., 1994; Porter et al., 1995). More specifically, His239 activates the Cys258 thiol for a nucleophilic attack at the carbonyl of Gly257, yielding an 18 kD N-terminal (Hh-N) fragment that presumably contains all the signaling functions, and a 25 kD C-terminal fragment (Hh-C) that apparently has no other function than to catalyze the cleavage. During this unusual autocatalytic cleavage a cholesterol moiety is covalently attached to the C-terminal part of Hh-N (Porter et al., 1996), making Hh proteins the only established examples of protein sterolation in contemporary biology. The fully processed N-terminal part of Hh is called Hh-Np. The cholesterol moiety is important for regulating the spatial distribution of the Hh signal (Burke et al., 1999; Ingham, 2001; The et al., 1999), anchoring Hh to biological membranes and thus hampering diffusion. Accordingly, inhibiting Hh sterolation in *Drosophila* resulted in a gain-of-function phenotype, Hh action being observed at place where normally signaling is not observed, suggesting improved diffusion (Burke et al., 1999; The et al., 1999). In limbs of rodent embryos containing sterolation-deficient Hh, however, digits close to the ZPA (zone of polarizing activity, the source of Hh in the limb bud) are normal but the more distant digits are absent, not in agreement with improved diffusion, but suggesting reduced diffusion. These seemingly contradictory results might be explained by a multitude of transportation mechanisms found to be able to deal with Hh’s hydrophobicity; for example the formation of Shh multimers in which the lipid attachments are sequestered in the interior of the multimer, making the complex soluble and freely diffusible (Zeng et al., 2001), nodal vesicular particles that consist of membrane fragments (Tanaka et al., 2005), lipoprotein-mediated trafficking as known for...
lipid transportation in blood (Panakova et al., 2005), and intracellular transportation acting like a relay race (described in more detail below).

Following the unusual posttranslational autocatalytic processing of the primary translated product, another acylation occurs. After cleavage, the Sonic Hh (Shh) protein is covalently palmitoylated at the N-terminal Cys24 site (Pepinsky et al., 1998). This is atypical as palmitoylation is usually restricted to the cytoplasm and thus only occurs on proteins without a signal peptide and not proteins entering the secretory vesicular pathway. Palmitoylated recombinant Hh has an approximately thirty-fold higher biological activity compared to unpalmitoylatable mutants (Pepinsky et al., 1998) suggesting that this acylation is important for enhanced biological activity. In accordance, in a Drosophila mutant lacking the acyltransferase that mediates Hh palmitoylation (the so-called sightless mutant (siti) the morphogen is functionally inactive (Chamoun et al., 2001; Lee and Treisman, 2001; Michelli et al., 2002). During mammalian development, however, although promoting biological activity the requirement of N-terminal acylation is certainly not absolute (Michelli et al., 2002). The main function of palmitoylation is the increase in hydrophobicity of the molecule and replacing groups that increase hydrophobicity increase Shh potency (Taylor et al., 2001). Possibly, the remarkable N-terminal acylation of Hh targets the molecule to lipid rafts, thereby facilitating the interaction with the Hh receptor Patched (Jeong and McMahon, 2002). Concluding, the exact functions of the Hh-N adducts are not yet clear but apparently the sterolation determines somehow the spatial distribution of Hh signaling but is not necessarily required for its biological activity.

The latter point, the lack of requirement per se of Hh sterolation for biological activity, is important in view of the deficiencies observed in patients with congenital abnormalities in cholesterol metabolism. In each of the six recognized sterol disorders, i.e., mevalonic aciduria, Smith-Lemli-Opitz syndrome, desmosterolosis, Conradi-Hunermann-Happle syndrome, CHILD syndrome, and Greenberg dysplasia, embryological abnormalities occur which are strikingly reminiscent to those observed with aberrant Hh signalling. Often these abnormalities are attributed to defective Hh processing, but this view is disputed by the aforementioned not-absolute requirement of sterolation for Hh biological activity. In agreement with this notion, it has recently been shown that in such developmental disorders endogenous cholesterol levels are sufficient to allow normal Hh processing, suggesting that deficient Hh sterolation is not fundamental for these abnormalities, and that the effects of diminished cholesterol metabolism are more downstream in the Hh signal transduction (Cooper et al., 2003). The finding that (pro-)vitamin D₃ is in fact the molecule responsible for inhibition of Smo (Smo) explains at least part of the puzzling phenotype of the Smith-Lemli-Opitz syndrome (Chapter 2 and (Bijlsma et al., 2006a; Bijlsma et al., 2006b)).

Importantly, unsterolated Hh may display disrupted intracellular transport, providing an alternative explanation for the birth defects in cholesterol metabolism disorders not explained by the inhibitory action of vitamin D₃. Recent evidence also demonstrates an intercellular action for sterolation in mediating proper intracellular transport of Hh proteins. This effect involves the Dispatched protein, a protein resembling a prokaryotic ion pump that transverses the membrane twelve times and that stimulates long range Hh signaling in Drosophila (Burke et al., 1999). In Drosophila melanogaster embryonic epithelia, cholesterol modification of Hh is responsible for its assembly into large punctate structures and apical sorting, apparently through the activity of the Dispatched protein (Gallet et al., 2005). Also in mammalian cells, Dispatched aids in transporting Hh via the cholesterol moiety over an as yet unidentified cellular membrane (Ma et al., 2002). In accordance, Dispatched knock-out mice closely resemble mice genetically deficient in more distal elements of Hh signaling (Kawakami et al., 2002). Hence, the cholesterol moiety in Hh seems essential for proper intracellular transport of Hh and for the correct extracellular concentration gradient. The sometimes-troublesome interpretation of genetic interference with Hh sterolation may be partly due to the complex interplay of these two effects. Furthermore, the action of Dispatched suggests that the acylations of Hh occur in the cytoplasm, before the protein enters the vesicular pathway via translocation by Dispatched, which provide a possible explanation as to how a palmitoylated protein localizes to the vesicular pathway.

**Transduction of the Hh Signal to its Targets**

The mechanisms of transduction of the Hh signal to its targets are only partly known, and important gaps in the biochemical details that mediate the action of the morphogen still need to be elucidated. Recently, however, our knowledge concerning Hh signaling has substantially increased and a schematic representation of the Hh pathway in the presence of Hh is shown in Fig. 3. It should be noted that large differences between vertebrate and arthropod model systems have become apparent and we will focus here on what is known about vertebrate Hh signal transduction.

The receptor for Hh is the 12-transmembrane protein called Patched (Ptch1). Binding of Hh to Ptch1 and its subsequent internalisation alleviates the inhibitory effect of Ptch1 on the 7-transmembrane protein Smo, which then activates the Hh pathway (Kalderon, 2000). Hh binding to
Ptch1 is equivalent to the absence (genetic loss) of Ptch1. The mechanism of Smo inhibition by Ptch1 was unknown at the start of the PhD project described in this thesis, but was suspected to be indirect since Ptch1 and Smo do not need to bind or co-localize, and Ptch1 also inhibits Smo when present sub-stoichiometrically (Taipale et al., 2002). Substantial homology exists between Ptch1 and the Niemann-Pick C1 (NPC1) protein, a protein involved in cholesterol homeostasis in humans, but also various prokaryotic transporter molecules (Davies et al., 2000). This homology gave rise to the conception that in the absence of Hh, Ptch1 pumps a compound out of the cell that binds to and inhibits Smo (Taipale et al., 2002). Indeed it was shown that Ptch1 translocates (pro-)vitamin D3 across the cell membrane, and that this vitamin D3 binds to Smo thereby inhibiting it (see Chapter 2 of this thesis and (Bijlsma et al., 2006a; Bijlsma et al., 2006b)).

Smo inactivation by antagonists like vitamin D3 is presumably achieved by lowering the phosphorylation state of Smo. This induces an intracellular conformational switch by which the cell surface expression is regulated in such a way that in an inactive state, hardly any Smo is present at the cell surface. (Kalderon, 2000; Zhao et al., 2007). Signaling downstream of Smo is still obscure in vertebrates but involves a complex of proteins that inhibits transcription factors from activating Hh targets. Signal transduction from Smo to this complex probably involves G-proteins, a notion that arises from the structural resemblance of Smo to G-protein coupled receptors, being a 7-pass transmembrane protein. Binding of G-proteins has been shown, as well as a necessity for this binding for activation of the pathway, but definitive studies need to confirm the actual role of the involvement of G-proteins in development (DeCamp et al., 2000; Riobo and Manning, 2005; Riobo et al., 2006).

The complex targeted by Smo presumably consists of the kinase Fused (Fu), Suppressor of Fused (SuFu), which is a negative regulator of Hh signaling and zinc finger transcription factors (glioma-associated oncogene, or Gli, protein family) (Nybakken et al., 2002; Robbins et al., 1997; Sisson et al., 1997; Stegman et al., 2000). Other proteins might be expected to associate with the complex to more closely mimic the situation in Drosophila, where Cos2 anchors the complex to the cytoskeleton. How the vertebrate complex interacts with the cytoskeletal will be discussed in more detail below. In the absence of a Hh signal, the above-mentioned complex functions to sequester the Gli transcription factors. There are three known Gli homologs in vertebrates, and they all differ in the way they respond to Hh. Gli1 is for instance only known to positively relay a Hh signal, and sequestration or proteolysis of Gli1 relates to “no response”. Gli2 on the other hand, seems to be able to exert two different functions; when full-length and activated, it is an activator of transcription of target genes, but when cleaved (in an inactive pathway) it acts as a repressor, inhibiting transcription of target genes. In this regard, Gli2 is most similar to the fruit fly cubitus interruptus (Ci) that is also capable of two modes of action. Gli3 seems only to be capable of translating an inactive pathway status in a repressor function, inhibiting the transcription of target genes (Riobo and Manning, 2007; Ruiz i Altaba et al., 2002; Wang et al., 2007).

Presumably, all of the Gli proteins (Dunaeva et al., 2003; Ingham and McMahon, 2001; Monnier et al., 2002; Nybakken and Perrimon, 2002) are phosphorylated by GSK-3β, PKA and CK1 (Doble and Woodgett, 2003; Jia et al., 2002; Lum et al., 2003), priming (in conjunction with b-TrCP, a ubiquitin ligase (Huntzicker et al., 2006)) them for

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**FIGURE 3. PROPOSED MECHANISM OF THE HH SIGNAL TRANSDUCTION PATHWAY IN THE PRESENCE OF HH**

In the absence of Hh ligand, Ptch1 secretes vitamin D3 into the extracellular medium to inhibit Smo. Binding of Hh to Ptch1 causes its inactivation and thus the secretion of vitamin D3 is blocked (indicated by cross). Smo is now active and signals to an inhibitory protein complex that keeps the Gli transcription factors from initiating transcription of target genes. The complex dissociates and following activation, Gli is imported into the nucleus and target genes are transcribed. Proteins not drawn to scale.
proteolysis (see (Riobo and Manning, 2007) for an exhaustive overview). These kinases fulfil a central role in controlling cellular physiology in all animals, usually activating proteins by phosphorylating them, but in this case the kinases have an inhibitory role. The involvement of GSK-3β in the inhibition of transcription factors is highly reminiscent of the Wnt pathway, in which Frizzled acts through Dishevelled to phosphorylate and inhibit GSK-3β (Barker and Clevers, 2000) and strikingly Smoothened is a close genetic relative of Frizzled. As GSK-3β is known to be an important negative regulator in the Wnt pathway, the convergence of Hh and Wnt signaling pathways on GSK-3β might mediate cross talk between both morphogenetic pathways.

Following Smoothened signaling through unclear mechanisms to the SuFu/Fu complex (Nybakken et al., 2002; Stegman et al., 2000), Gli is no longer subject to phosphorylation and sequestration. Subsequently, Gli is activated and this free, full length activated Gli translocates into the nucleus. Upon binding to the proper DNA consensus sequences it activates transcription of Hh targets and a Hh response ensues.

Recently, it has become clear that much of the Hh signaling machinery localizes to the primary cilium, a structure present on most cell surfaces consisting of microtubules (Corbit et al., 2005; Rohatgi et al., 2007). Apparently, Ptch1 regulates the position of Smo in the cilium, thus affecting its capacity to signal to the Gli transcription factors also found in the cilium. Mutations that abrogate the cilium also affect Hh signaling capacity and it is thought that the role of ciliary localization of Hh signaling components is a physiologically relevant phenomenon (Zhang et al., 2005).

New Hh homologues
In 1993, a joint effort of three research groups resulted in the identification of vertebrate Hh genes, each rather comically named (Echelard et al., 1993; Krauss et al., 1993; Riddle et al., 1993). Using primary sequence comparisons of these homologues in various species, clues can be obtained as to the evolutionary history of this protein family. The fact that Hh homologues were recovered from both insect as well as vertebrate genomes (Fig. 4) demonstrates that these morphogens were already present in ancestral bilaterian living before the Cambrian explosion (over 545 million years ago).

Following the cloning of the murine Hh homologues, it was shown that Shh mediates the action of the ZPA in the chick limb bud (Riddle et al., 1993). The ZPA is a region at the posterior margin of the chick limb bud responsible for normal anteroposterior patterning. Hh expression co-localized with this previously described region and ectopic expression of Hh can alter limb patterning in a way similar to ZPA grafting. Also, Hh was shown to activate the expression of the cell identity, determining Hoxd genes in the same fashion as ZPA grafts do. Thus Shh is the responsible molecule mediating ZPA action. At the time the principal role of Shh in ZPA action was highly controversial, as the morphogenetic activity of the ZPA was generally considered to be retinoic acid-mediated. The observation, however, that retinoic acid induced Shh expression and that Shh was sufficient to explain retinoic acid effects on developing limb gene expression suggested that Shh is the relevant polarising signal in the ZPA (Riddle et al., 1993) and today this notion is uncontested.

Although Shh is the most important mammalian Hh isoform, both Ihh and Dhh are also implicated in a variety of embryonic, physiological and pathological processes. Ihh regulates proliferation and differentiation of chondrocytes, regulates pancreatic development and is expressed in the adult kidney and large intestine, whereas Dhh is expressed in adult testis. The functionality of these adult expression patterns is not very well understood, but influencing signaling of these proteins in diseases like osteoporosis and diabetes (which are becoming increasingly problematic in the ageing Western societies) might be an interesting option for therapeutic treatment.

Hh target genes
Following the relief of transcriptional repression, Hh target genes will be transcribed. These target genes used to encompass a relatively limited set of proteins of which HNF3β, Hox genes, various BMP morphogens, Ptc1 and HIP (Hh Inhibitory Protein; an extracellular Hh inhibitor (Chuang and McMahon, 1999)) were the most prominent members. Remarkably, the upregulation of Ptc1 and HIP evidently provide feed back on the Hh signal, and thus Hh immediately downregulates its own activity, either via the expression of the inhibitory component of its receptor or via inhibitory proteins. HNF3β, the Hox genes and the various
BMP morphogens are obviously implicated in transducing the morphogenetic signal. The advent of the micro array technique, however, has greatly complicated matters and for reports on the diverse set of Hh target genes, see references (Ingram et al., 2002; Kato et al., 2001; Pola et al., 2001; Yoon et al., 2002). Hh target genes found by micro array analysis include molecules involved in cell cycle (Yoon et al., 2002), cell adhesion (Yoon et al., 2002), signal transduction (Yoon et al., 2002), apoptosis (Ingram et al., 2002; Yoon et al., 2002), nerve formation (Ingram et al., 2002), transcriptional regulation (Ingram et al., 2002), Wnt signaling antagonism (Ingram et al., 2002), protease inhibition and metal binding (Kato et al., 2001). This abundance of targets reflects at least to some extent the versatile role of Hh signaling throughout the body. The actual in vivo relevance of these genes remains largely unclear and awaits functional studies for further validation.

**EXPRESSION AND FUNCTION OF THE MAMMALIAN Hh PROTEINS IN NORMAL HEALTH AND TUMORIGENESIS**

In situ hybridisation and immunohistochemical studies showed that all three mammalian Hh homologues displayed intricate expression patterns during development as well as in adult organisms. Most well known is the expression of Shh in ZPA and its subsequent role in digit formation, which is conserved throughout all vertebrates. However, virtually all pattern forming events, but especially those involving epithelia, prominently feature Shh signaling (e.g. tooth development, neuronal development and pattern formation along axial midline; excellently reviewed by Ingham (Ingham and McMahon, 2001)) and thus Shh can be argued to be the most significant of the three mammalian Hh homologues.

Interestingly, recently it has become clear that Shh signaling remains active in the adult, maintaining tissue architecture in face of the persistent sequence of proliferation, differentiation and apoptosis that challenges the shape and integrity of tissue in the gut. Initially, during embryonic development, Shh is expressed in the endoderm throughout the gut tube, however, in the mouse from 10.5 days post coitum on, this expression is gradually restricted to the glands of the proximal stomach and the base of the villi in the intestine (Bitgood and McMahon, 1995; Ramalho-Santos et al., 2000). In the crypts of the fundic stomach, Shh is expressed in the endoderm and remains the only established example of a stereotyped protein within the animal kingdom. Its signaling is truly unique, as the dual receptor system of Ptc1 and Smo gives rise to the seemingly contradictory situation that increased receptor expression gives rise to diminished signalling. Signaling downstream of Smo remains obscure but involves a variety of unusual mechanisms, including a series of sequential inhibitions. These idiosyncratic features may reflect its ancient evolutionary past, while highlighting its special interest for physiology.

**CONCLUDING REMARKS**

Of the network of mediates morphogenetic signals in development and adult physiology, Hh signaling remains one of the least understood and seems to have some unique features: the protein is palmitoylated while being transported in the vesicular secretory pathway (not residing in the cytoplasm) and remains the only established example of a stereotyped protein within the animal kingdom. Its signaling is truly unique, as the dual receptor system of Ptc1 and Smo gives rise to the seemingly contradictory situation that increased receptor expression gives rise to diminished signalling. Signal downstream of Smo remains obscure but involves a variety of unusual mechanisms, including a series of sequential inhibitions. These idiosyncratic features may reflect its ancient evolutionary past, while highlighting its special interest for physiology.

**ACKNOWLEDGEMENTS**

The authors’ research is supported by grants from STW and NWO.

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