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Palmitoylation of Hedgehog Proteins

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Abstract

Hedgehog (Hh) proteins are secreted signaling proteins that contain amide-linked palmitate at the N-terminus and cholesterol at the C-terminus. Palmitoylation of Hh proteins is critical for effective long- and short-range signaling. The palmitoylation reaction occurs during transit of Hh through the secretory pathway, most likely in the lumen of the ER. Attachment of palmitate to Hh proteins is independent of cholesterol modification and autoprocessing and is catalyzed by Hhat (Hedgehog acyltransferase). Hhat is a member of the membrane bound *O*-acyltransferase (MBOAT) family, a subgroup of multipass membrane proteins that catalyze transfer of fatty acyl groups to lipids and proteins. Several classes of secreted proteins have recently been shown to be substrates for MBOAT acyltransferases, including Hh proteins and Spitz (palmitoylated by Hhat), Wg/Wnt proteins (modified with palmitate and/or palmitoleate by Porcupine) and ghrelin (octanoylated by ghrelin *O*-acyltransferase). These findings highlight protein fatty acylation as a mechanism that not only influences membrane binding of intracellular proteins but also regulates the signaling range and efficacy of secreted proteins.

I. Introduction to Protein Palmitoylation

Protein palmitoylation refers to the posttranslational modification of a protein with the 16-carbon fatty acid palmitate. In the majority of palmitoylation reactions, the palmitate donor is palmitoyl CoA (Resh, 2006; Smotrys and Linder, 2004). The term protein palmitoylation can also encompass posttranslational modification of proteins with other long chain fatty acids, as many palmitoylated proteins have been observed by mass spectrometry to incorporate heterogeneous mixtures of fatty acids at their modification site (Liang *et al*., 2001, 2004). In nearly all cases, palmitate is attached to proteins via thioester linkage to a cysteine residue; this is known as *S*-palmitoylation. The labile nature of the thioester linkage in *S*-palmitoylated proteins allows for consecutive rounds of palmitoylation/depalmitoylation/repalmitoylation in a fashion similar to phosphorylation (Goodwin *et al*., 2005; Loisel *et al*., 1999; Lorentzen *et al*., 2010; Yeh *et al*., 1999). A small subset of palmitoylated proteins, including the *Drosophila* epidermal growth factor (EGF) like ligand Spitz (Miura *et al*., 2006) and Hedgehog (Hh) family members (Chamoun *et al*., 2001; Pepinsky *et al*., 1998) are palmitoylated on a cysteine residue that is the N-terminal residue of the mature protein. *N*-Palmitoylation, in contrast to *S*-palmitoylation, is a stable modification. Palmitate attached via amide linkage is essentially as stable and long lived as

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the polypeptide backbone peptide bond. *N*-Palmitoylation can be readily distinguished from *S*-palmitoylation via its stability in neutral buffered hydroxylamine. The thioester linkage in *S*-palmitoylated proteins is hydrolyzed by hydroxylamine treatment while the amide linkage in *N*-palmitoylated proteins is resistant to hydrolysis.

Classes of proteins known to be modified with palmitate include cytoplasmic signaling molecules such as Src family tyrosine kinases (Alland *et al.*, 1994), the Ras family of small GTPases (Hancock *et al.*, 1989), heterotrimeric G-proteins (Kleuss and Krause, 2003), endothelial nitric oxide synthase (E-NOS) (Yeh *et al.*, 1999), and scaffolding proteins (Zhang *et al.*, 1998). Palmitoylation also occurs on several classes of membrane embedded proteins including ion channels (Gubitosi-Klug *et al.*, 2005; Qin *et al.*, 1998), transporters (Singaraja *et al.*, 2009), receptors, and tetraspanins (Resh, 2006). Finally, in addition to intracellular and membrane embedded proteins, palmitate has also been shown to be attached to secreted signaling molecules including Spitz (Miura *et al.*, 2006), members of the Wnt/wingless (Wg) family (Kurayoshi *et al.*, 2007; Willert *et al.*, 2003; Zhai *et al.*, 2004), as well as *Drosophila* and mammalian Hh family members (Buglino and Resh, 2008; Chamoun *et al.*, 2001; Pepinsky *et al.*, 1998).

Modification with palmitate has been implicated in the regulation of protein trafficking and localization, signal transduction, and enzymatic function. In general, attachment of palmitate increases the hydrophobicity of the modified protein and promotes membrane binding. Palmitoylation also enhances the partitioning of many signaling molecules into lipid rafts. Rafts are liquid ordered microdomains within cellular membranes, and raft association is implicated in mediating efficient signal transduction (Arcaro *et al.*, 2001; Fragoso *et al.*, 2003; Zhang *et al.*, 1998).

II. Palmitoylation of Hedgehog Proteins

The Hh family is the best studied example of palmitoylated, secreted signaling molecules. *Drosophila* express a single Hh protein, known simply as Hedgehog (Hh) (Nusslein-Volhard and Wieschaus, 1980). There are three Hh family members in mammals, Sonic Hedgehog (Shh), Indian Hedgehog (Ihh), and Desert Hedgehog (Dhh) (Bitgood and McMahon, 1995; Echelard *et al.*, 1993). Signaling by Hh family members plays a major role in embryonic patterning in both flies and mammals (Echelard *et al.*, 1993; Ericson *et al.*, 1995; Heemskerk and DiNardo, 1994; Ma *et al.*, 1993). All Hh family members are thought to be palmitoylated; however, direct incorporation of palmitate to hedgehog in cells has only been documented for Hh and Shh (Buglino and Resh, 2008; Pepinsky *et al.*, 1998). Palmitoylation comprises one of a series of modifications that occur on Hh proteins, as detailed below.

A. Hedgehog biosynthesis, autoprocessing, and cholesterol incorporation

All Hh family members are initially synthesized as an ~45-kDa precursor protein (Fig. 10.1). The precursor contains an amino-terminal signal sequence that directs entry into the endoplasmic reticulum (Lee *et al.*, 1992). Upon cleavage of the signal sequence, Hh is further processed in the ER by an autoproteolysis reaction catalyzed by the C-terminal half of the molecule (Chen *et al.*, 2011; Lee *et al.*, 1994). Hh autoprocessing occurs via a mechanism similar to that of intein processing in self-splicing proteins (Hall *et al.*, 1997).

Autoprocessing requires an intact C-terminal domain and can be blocked by mutation of the histidine residue at position 329 or mutation of the cleavage site cysteine (C258) (Porter *et al.*, 1995, 1996a). Shh autoprocessing can also be inhibited by depletion of cellular sterols (Guy, 2000).

The Shh cleavage reaction proceeds via a two-step mechanism. First, the sulfhydryl group of an invariant cysteine residue acts as a nucleophile to attack the carbonyl of the preceding glycine residue resulting in the formation of a thioester intermediate. This thioester linkage is then subject to nucleophilic attack by the 3β-hydroxyl group of a cholesterol molecule, resulting in the liberation of the C-terminal autoprocessing domain and the formation of an ester link between a cholesterol moiety and the carboxyl-terminal glycine residue of the 19-kDa amino-terminal fragment (Porter *et al.*, 1996a,b). Cholesterol is the only nucleophile whose incorporation into Hh has been directly observed *in vivo*. However, other related sterols can replace cholesterol in the autoprocessing reaction *in vitro* with varying degrees of efficiency (Cooper *et al.*, 1998). From *in vitro* assays, it is clear that the most important structural feature for autoprocessing is the C3 hydroxyl moiety. This group must be free of esterified adducts and there is an absolute requirement for the β orientation of the alcohol (Cooper *et al.*, 1998). The 19-kDa amino-terminal domain of Hh mediates all known signaling functions of Hh, while the C-terminal autoprocessing domain is primarily responsible for catalyzing autoprocessing and cholesterol incorporation (Fietz *et al.*, 1995; Marti *et al.*, 1995; Porter *et al.*, 1995). Following autocleavage, the C-terminal Shh fragment is rapidly degraded in the ER lumen by the ERAD (ER-associated degradation) pathway (Chen *et al.*, 2011).

B. Hedgehog palmitoylation

A molecule of palmitate is covalently attached via amide linkage to the amino-terminal cysteine residue of both Hh and Shh (Pepinsky *et al.*, 1998). The 19-kDa amino-terminal fragment of Hh/Shh containing both a C-terminal cholesterol and a N-terminal palmitate represents the mature signaling molecule and is the predominant form of Hh/Shh secreted *in vivo* (Taipale *et al.*, 2000). Shh palmitoylation is catalyzed by Hhat (Hedgehog acyltransferase), and this reaction can be recapitulated in cells and *in vitro* (Buglino and Resh, 2008, 2010).

Palmitoylation of Shh in cells occurs following cleavage of the signal sequence. Cotransfection of mammalian cells with cDNAs encoding Shh and Hhat reveals several features of the Shh palmitoylation reaction. Shh and Hhat colocalize to the endoplasmic reticulum and the Golgi (Buglino and Resh, 2008). Transit through the secretory pathway is essential, as Shh constructs that lack the N-terminal signal sequence are not palmitoylated (Buglino and Resh, 2008). These findings suggest that palmitoylation of Shh by Hhat occurs intracellularly, in the lumen of secretory organelles. Once the signal sequence is cleaved, cysteine becomes the N-terminal residue and this is the site of modification (Fig. 10.2). Palmitoylation is thought to initially occur via thioester linkage to the cysteine residue. The thioester intermediate then rearranges to an amide linkage via an intramolecular S-to-N shift, producing an amide or N-linked palmitate (Fig. 10.2A) (Mann and Beachy, 2004; Pepinsky *et al.*, 1998). However, in this scenario, the cysteine sulfhydryl group would be regenerated.

Hhat could attach a second palmitate via a thioester bond, and a thioesterase would be needed to remove the second, thioester linked fatty acid. An alternative mechanism is direct attachment of the palmitate moiety to the N-terminal amide via amide linkage (Fig. 10.2B), analogous to *N*-myristoylation (Farazi *et al.*, 2001). This model is supported by the findings that N-terminally blocked Shh proteins are not substrates for Hhat and that thioester linked palmitoylated intermediates of Shh cannot be detected (Buglino and Resh, 2008). Further studies will be required to define the exact reaction mechanism.

An N-terminal cysteine is required for Shh palmitoylation. Shh mutants with substitutions of either alanine or serine have vastly reduced levels of palmitate incorporation. Signal sequence removal by signal peptidase is therefore absolutely essential as this cleavage event generates the N-terminal cysteine. Attachment of palmitate has been documented on both the 45 kDa Shh precursor protein as well as on the mature 19 kDa form of Shh. Moreover, a Shh mutant that is defective in autoprocessing and cholesterol attachment can still efficiently incorporate palmitate (Buglino and Resh, 2008). Thus, the two lipidation reactions occur independently.

C. Rasp and Hhat are Hh/Shh palmitoyl acyltransferases

Genetic studies in *Drosophila melanogaster* suggested that palmitoylation of Hh was catalyzed by a member of the membrane bound *O*-acyltransferase (MBOAT) family that was independently identified as Skinny Hedgehog, Sightless Hedgehog, Central missing, or Rasp (Amanai and Jiang, 2001; Chamoun *et al.*, 2001; Lee and Treisman, 2001; Micchelli *et al.*, 2002). Rasp is required for induction of Hh target genes and proper patterning of both the *Drosophila* wing and eye imaginal disks, as well as for proper segmentation of *Drosophila* larva (Chamoun *et al.*, 2001; Lee and Treisman, 2001; Micchelli *et al.*, 2002). In addition, the *rasp* null phenotype closely phenocopies mutation of the palmitate acceptor site cysteine within Hh in the wing imaginal disk. Rasp activity is essential in cells of the posterior compartment that produce Hh but is not required for Hh transcription or secretion, leading to the stipulation that Rasp functions instead in the maturation of Hh signal (Chamoun *et al.*, 2001; Lee and Treisman, 2001; Micchelli *et al.*, 2002). Rasp mutant organisms display defects in Hh signaling independent of both autoprocessing and cholesterol incorporation, implying that Rasp does not play a role in these processes (Amanai and Jiang, 2001). Additional evidence that Rasp regulates Hh palmitoylation stems from the observation that Hh is significantly less hydrophobic when isolated from rasp null cells than when isolated from WT cells (Chamoun *et al.*, 2001).

The human homologue of Rasp, originally termed Skinny hedgehog, is known as Hhat (*Hedgehog acyltransferase*). Loss of Hhat function in the mouse closely phenocopies the mutant phenotype resulting from mutation of the palmitoylation site within Shh when assayed in either the limb or neural tube (Chen *et al.*, 2004). In addition, [^{3}H]-palmitate labeling of Shh is reduced when Shh is isolated from Hhat null murine embryonic fibroblasts (MEF)'s, consistent with Hhat's putative role in Shh palmitoylation (Chen *et al.*, 2004). Palmitoylation of Hh/Shh depends on the presence of a cysteine residue (position 85 in *Drosophila*, position 24 in human Shh, and position 25 in mouse Shh) immediately following the signal peptide cleavage site and on a functional form of Hhat or Rasp

(Chamoun *et al.*, 2001; Pepinsky *et al.*, 1998). A Rasp construct with alanine substituted for both the aspartate at position 341 and the histidine at position 381 in tandem is unable to rescue the *rasp* mutant phenotype, implicating these residues in Rasp activity (Chamoun *et al.*, 2001).

Definitive evidence that Hhat is a palmitoyl acyltransferase for Shh was achieved when Hhat was purified to homogeneity (Buglino and Resh, 2008). Incubation of purified Hhat with purified, recombinant Shh results in near-stoichiometric incorporation of palmitate onto the N-terminal cysteine via amide linkage, features that recapitulate Shh palmitoylation in cells. The reaction occurs catalytically, uses palmitoyl CoA as the palmitate donor, and exhibits optimal activity at pH 6.5. Hhat appears to be relatively specific for Shh, as other palmitoylated proteins (H-Ras, PSD-95, Wnt proteins) are not substrates for palmitoylation by Hhat *in vitro*. A peptide containing the first 11 amino acids of the mature Shh sequence is a substrate for Hhat-mediated palmitoylation *in vitro*, thereby defining a minimal sequence for Shh palmitoylation (Buglino and Resh, 2008). In addition, the Shh substrate must contain an N-terminal cysteine with a free amino terminus, as purified Hhat cannot palmitoylate Shh peptides or proteins if the N-terminus is blocked by acetylation or by a hexa-histidine tag.

D. Role of palmitoylation in Hh signaling

The Hh-signaling cascade is utilized throughout development. Hh signaling plays a significant role in patterning of many aspects of the mammalian body plan, including the developing neural tube, limbs, axial skeleton, and induction of ventral forebrain structures (Echelard *et al.*, 1993; Ekker *et al.*, 1995; Ericson *et al.*, 1995; Lopez-Martinez *et al.*, 1995; Roelink *et al.*, 1995). Loss of Hh-signaling activity during vertebrate embryogenesis causes severe developmental disorders such as holoprosencephaly, craniofacial defects, and skeletal malformations (Cooper *et al.*, 1998; Incardona and Roelink, 2000; Lee *et al.*, 2001). In addition to its role in development, Hh has been implicated in the regulation of tissue homeostasis, stem cell biology, and the progression of many types of human malignancies, most notably mebdulloblastoma, pancreatic cancer, and other cancers of the GI tract (Feldmann *et al.*, 2007; Karhadkar *et al.*, 2004; Tian *et al.*, 2009; Yauch *et al.*, 2008).

Palmitoylation of Hh is required for proper signaling in flies. Mutation of the acceptor site cysteine produces a protein that has little to no patterning activity in *Drosophila* (Dawber *et al.*, 2005; Lee *et al.*, 2001). Expression of Hh C85S is not sufficient for rescue of the Hh phenotype of *Drosophila* larva despite the fact that it is expressed at levels similar to WT Hh and is properly autoprocessed. Misexpression of WT Hh in the posterior compartment of the wing disk leads to expansion of the anterior compartment, expanded expression of Hh target genes Ptc and Dpp, and patterning defects in the adult wing. Misexpression of Hh C85A, however, does not result in expansion of the anterior compartment or expanded expression of Patched and dpp in the wing disk, indicating that it is severely attenuated in signaling capacity (Lee *et al.*, 2001).

Mutation of the acceptor cysteine also results in a reduction of Shh signaling in mammalian tissues. However, nonpalmitoylated forms of Shh appear to be more active than their *Drosophila* counterparts. This is evidenced by the observation that ectopic overexpression of

Shh C25S is still able to cause polydactyly in the mouse limb (Lee *et al.*, 2001). However, the signaling activity of nonpalmitoylated forms of Shh is clearly reduced in both the mouse limb and neural tube (Chen *et al.*, 2004). In addition, injection of retroviruses encoding WT Shh into the forebrains of E9.5 rats results in severe brain deformities, while retroviruses that encode nonpalmitoylated forms of Shh are unable to cause such defects (Kohtz *et al.*, 2001).

Studies in the mouse embryonic fibroblast line, C3H10T1/2, have revealed that palmitoylation also affects Shh potency *in vitro*. C3H10T1/2 is a mesenchymal cell line that can be induced under defined conditions to differentiate into adipocytes, chondrocytes, or bone osteoblasts (Pepinsky *et al.*, 1998). Upon differentiation into the bone lineage, there is a marked upregulation of alkaline phosphatase activity, which can be used as a marker for this process (Pepinsky *et al.*, 1998). Treatment of C3H10T1/2 cells with Shh causes a dose-dependent increase in alkaline phosphatase activity and is frequently used as a readout of Shh activity *in vitro*. Palmitoylated forms of Shh are 40–160-fold more active compared to unmodified Shh in this assay (Pepinsky *et al.*, 1998). The enhanced potency of palmitoylated forms of Shh does not correlate with enhanced receptor binding, as palmitoylated and nonpalmitoylated forms of Shh bind equally well to cells expressing Ptc (Pepinsky *et al.*, 1998). The hydrophobic nature of palmitate appears to directly contribute to its ability to enhance Shh-signaling potency. Increasing the hydrophobic character of the amino terminus of Shh, either by introducing a stretch of hydrophobic amino acids or by chemical modification, results in increased potency over WT unmodified forms. By contrast, introduction of hydrophilic residues at the N-terminus of Shh results in reduced signaling in a cell-based differentiation assay (Taylor *et al.*, 2001).

Release of Hh/Shh into the media requires the action of Dispatched, a 12-transmembrane (TM) domain containing protein (Burke *et al.*, 1999). Active Hh/Shh is released from the producing cell as a multimeric protein, and this process is facilitated by multimerization of Hh proteins on the cell surface. Dual lipidation enables Hh proteins to form nanoscale oligomers that colocalize with heparin sulfate proteoglycans (HSPGs) (Vyas *et al.*, 2008). Association with HSPGs promotes interaction of Hh proteins with ADAM17, a metalloprotease that has been implicated in Hh shedding from the cell surface (Dierker *et al.*, 2009). A recent study suggests that palmitoylation helps to position the N-terminal region of Shh for ectodomain cleavage by ADAM17 (Ohlig *et al.*, 2011). Proteolytic removal of the palmitoylated N-terminus is proposed to be required for binding of activated Shh to Patched.

After release from the cell, Hh family members act as morphogens that signal in a concentration-dependent manner (Fuccillo *et al.*, 2006; Gritli-Linde *et al.*, 2001; Heemskerk and DiNardo, 1994; Stamataki *et al.*, 2005). Hh responsive cells adopt specific cell fates, or induce different transcriptional profiles, in part depending upon the level of Hh signal received (Gritli-Linde *et al.*, 2001; Heemskerk and DiNardo, 1994). Hh protein levels and, therefore, signaling are highest at sites of Hh synthesis and decay as the distance between the source of Hh and the responding cell increases. Forming and maintaining the Hh-signaling gradient is essential for proper patterning of the cuticle in Drosophila, as well as the neural tube and distal limb elements during mammalian development (Gritli-Linde *et al.*,

2001; Heemskerk and DiNardo, 1994; Lee *et al.*, 2001). Posttranslational lipophilic modifications of the Hh ligand have important effects on the Hh-signaling gradient. Lipophilic modification influences partitioning of Hh proteins into lipoprotein particles, which have been implicated as playing an important role in long range Hh signaling (Callejo *et al.*, 2008; Panakova *et al.*, 2005). Modification of Hh with cholesterol and palmitate is also required for the formation of soluble multimeric forms of Hh that are freely diffusible, accumulate in a gradient, and enable the molecule to signal over long distances (Chen *et al.*, 2004; Goetz *et al.*, 2006; Zeng *et al.*, 2001).

E. Role of cholesterol modification and autoprocessing in Hh signaling

Autoprocessing and cholesterol incorporation into Hh/Shh are essential for proper tissue distribution and patterning activity. This is evidenced by the observation that several point mutations linked to holoprosencephaly occur within regions of Shh that have been implicated in autoprocessing and cholesterol incorporation (Maity *et al.*, 2005; Roessler *et al.*, 2009). However, Hh/Shh constructs that do not incorporate cholesterol still retain significant signaling activity over both short and long distances (Dawber *et al.*, 2005).

In the initial study characterizing Hh autoprocessing, ectopic overexpression of a cleavage defective form of Hh (H329A) still induced expanded Wg expression during *Drosophila* embryonic development, although to a lesser degree than when a WT construct was expressed (Lee *et al.*, 1994). This finding suggests that unprocessed Hh is able to signal but in a reduced capacity. Unprocessed forms of Hh were also less active in patterning of dorsal cuticle structures, and in patterning of the wing and eye imaginal disks (Lee *et al.*, 1994; Porter *et al.*, 1995). More recent studies have found that full-length unprocessed Hh is trafficked to the plasma membrane and can participate in direct cell-to-cell signaling, both *in vivo* and *in vitro*, but is unable to act over longer distances (Tokhunts *et al.*, 2009).

The reduction in signaling potential of unprocessed forms of Hh results from failure to liberate the amino-terminal signaling domain. Overexpression of Hh-N (the 19-kDa N-terminal fragment of Hh unmodified by cholesterol) alone is able to induce similar levels of Wg expression and causes nearly identical changes in dorsal cuticle patterning as full-length Hh (Burke *et al.*, 1999; Porter *et al.*, 1995). Hh-N is also able to rescue most Hh function in the wing imaginal disk (Burke *et al.*, 1999; Dawber *et al.*, 2005). Similarly, Shh-N still retains patterning activity in the mouse limb bud and chick neural tube (Caspary *et al.*, 2002; Yang *et al.*, 1997). In addition, purified recombinant Shh-N constructs lacking cholesterol are still able to bind to Ptc expressing cells and activate Shh signaling *in vitro* (Taylor *et al.*, 2001). When tested in the C3H10T1/2 differentiation assay, Shh-N lacking cholesterol induced alkaline phosphate upregulation but to a lesser degree than WT Shh (Taylor *et al.*, 2001). Taken together, these findings indicate that modification of Hh/Shh with cholesterol enhances signaling potency but is not strictly required for signaling activity *in vitro* or *in vivo*.

Incorporation of cholesterol into Hh/Shh regulates proper tissue distribution during development. Cholesterol incorporation into Hh/Shh restricts diffusion and promotes tethering of the molecule to membranes (Pepinsky *et al.*, 1998). Restriction of Hh/Shh diffusion is essential for forming a steep concentration gradient. Regulated overexpression

of Hh-N using the GAL4:UAS system results in higher levels of Wg expression and causes more severe defects in dorsal cuticle patterning when compared to full-length Hh (Porter *et al.*, 1996a). This increased activity was correlated with a more diffuse staining pattern and reduced hydrophobic character of Hh-N, consistent with the cholesterol moiety acting to restrict the spread of Hh over long distances and promoting high levels of signaling near the site of synthesis. Similarly, reduced spread of cholesterol-modified forms of Hh has also been observed in the *Drosophila* wing disk (Dawber *et al.*, 2005).

Several lines of evidence suggest that cholesterol incorporation also restricts Shh diffusion in higher eukaryotes. The interaction of Hh with both Dispatched and HSPGs is dependent on cholesterol modification of Hh (Bellaiche *et al.*, 1998; Burke *et al.*, 1999; Kawakami *et al.*, 2002). Cholesterol incorporation has been shown to restrict the spread of Shh within the mouse limb bud (Li *et al.*, 2006). Shh-N constructs were distributed over a broader range and displayed reduced signaling potency within the limb bud when compared to full-length Shh (Li *et al.*, 2006). Similarly, when Shh-N expressed from COS-7 cells was grafted into the chick wing, it was able to diffuse further from the graft site compared to full-length Shh (Yang *et al.*, 1997). Expression of full-length human Shh in high five insect cells produces a cholesterol modified amino-terminal signaling domain which is primarily associated with the cell membrane (Pepinsky *et al.*, 1998). Mass spectrometry analysis of the minor soluble fraction of Shh remaining revealed it to be unmodified by cholesterol (Pepinsky *et al.*, 1998). Taken together, these results indicate that modification of Hh/Shh with cholesterol promotes membrane binding and favors short-range signaling. However, it is clear that cholesterol modified forms of Shh are able to directly activate signaling at a distance, and in some cases cholesterol appears to enhance long-range signaling (Briscoe *et al.*, 2001; Wang *et al.*, 2000). Thus, the question arises: how can a hydrophobic modification be required for both short- and long-range signaling?

Two possible mechanisms have been proposed to explain the requirement of cholesterol for efficient long-range Hedgehog signaling. First, cholesterol incorporation into Shh has been implicated in the formation of soluble multimeric forms of Shh (Zeng *et al.*, 2001). Second, cholesterol modification influences the partitioning of Hh into lipoprotein particles (Panakova *et al.*, 2005). Both of these structures would be able to travel longer distances and mediate long-range signaling.

III. Palmitoylation of Other Secreted Proteins

In addition to Hh family members, at least two other families of secreted signaling molecules have been shown to be palmitoylated: *Drosophila* EGF receptor (EGFR) ligands and members of the Wnt/Wg family. In both cases, palmitoylation is important for the function of the modified proteins.

A. EGF receptor ligands in flies

Spitz is a *Drosophila* EGFR ligand that is required for normal embryonic development, influencing the patterning of the eye, wing, and leg imaginal disks (Freeman, 1994; Miura *et al.*, 2006; Rutledge *et al.*, 1992). Spitz is initially synthesized as a membrane tethered precursor that is secreted following intracellular cleavage by the protease Rhomboid (Urban

et al., 2001). The mature secreted Spitz molecule is palmitoylated on an N-terminal cysteine residue and it is therefore likely that the palmitate attached to Spitz is amide linked (Miura *et al.*, 2006). Once secreted, palmitoylation of Spitz promotes its association with the extracellular face of the plasma membrane and acts to concentrate Spitz locally at the site of secretion (Miura *et al.*, 2006). This restriction of Spitz diffusion sets up a sharp concentration gradient at the site of synthesis. Palmitoylation is essential for proper Spitz signaling *in vivo*; nonpalmitoylated forms of Spitz are unable to rescue photoreceptor differentiation. However, palmitoylation of Spitz is not required for receptor binding or activation, as nonpalmitoylated Spitz signals in a fashion indistinguishable from palmitoylated Spitz *in vitro* (Miura *et al.*, 2006). Two other *Drosophila* EGF ligands, Keren and Gurken, also contain amino-terminal cysteine residues and are therefore also predicted to be *N*-palmitoylated (Miura *et al.*, 2006). However, direct incorporation of palmitate into either Keren or Gurken has not yet been demonstrated.

The acyltransferase responsible for Spitz palmitoylation is Rasp (Miura *et al.*, 2006). Mutation of Rasp impairs Spitz signaling in both the eye and wing imaginal disks (Miura *et al.*, 2006). Reduction of rasp mRNA levels by siRNA knockdown reduces the hydrophobic character of Spitz as judged by Triton X-114 detergent phase separation (Miura *et al.*, 2006). In addition, palmitoylation of Spitz in a heterologous system (COS-1 cells) is dependent on coexpression of a functional clone of Rasp (Miura *et al.*, 2006). Taken together, these results support the hypothesis that Rasp mediates Spitz palmitoylation.

B. Wnt and Wg proteins

Palmitoylation regulates signaling by members of the Wnt/Wg family of signaling molecules. Wnt/Wg family members are secreted glycoproteins that play important roles during development as well as in the progression of many types of human malignancies (Beachy *et al.*, 2004). Wnt/Wg proteins are heterogeneously glycosylated and palmitoylated (Tanaka *et al.*, 2002; Willert *et al.*, 2003). Palmitoylation has been directly observed in the case of Wg, Wnt3a, Wnt5a, and Wnt8 (Doubravska *et al.*, 2011; Kurayoshi *et al.*, 2007; Willert *et al.*, 2003; Zhai *et al.*, 2004). Murine Wnt3a is palmitoylated on Cysteine 77 (Willert *et al.*, 2003). This position is conserved in nearly all Wnt proteins, suggesting that other Wnt family members are also likely palmitoylated.

Modification of Wnt3a by palmitate is not essential for secretion or glycosylation of Wnt3a but is required for proper signaling. Both WT and C77A Wnt-3a (a non-palmitoylated mutant) can be efficiently recovered from conditioned media produced by transfected tissue culture cells (Willert *et al.*, 2003). However, non-palmitoylated Wnt3a is compromised in its ability to bind its receptors LRP6 and Frizzled 8 (Komekado *et al.*, 2007). In addition, purified Wnt3a C77A is unable to induce β-catenin stabilization when added to mouse L cells, indicating that palmitoylation is essential for Wnt3a signaling (Willert *et al.*, 2003). Palmitoylation serves similar functions in the case of Wnt5a. Preventing palmitoylation of Wnt5a reduces receptor binding and compromises downstream signaling but appears dispensable for secretion (Kurayoshi *et al.*, 2007). By contrast, palmitoylation of *Drosophila* Wg at position 93 is essential for Wg secretion. Treatment of *Drosophila* S2 cells with the inhibitor of protein palmitoylation, 2-bromopalmitate prevents efficient Wg secretion into

the culture media (Zhai *et al.*, 2004). In addition, mutation of Cys 93 to Ala compromises secretion of Wg in the wing imaginal disk (Franch-Marro *et al.*, 2008). The C93A Wg mutant accumulates within the ER, indicating that palmitoylation is essential for intracellular trafficking and secretion of Wg (Franch-Marro *et al.*, 2008).

Wnt3a is also modified with a monounsaturated fatty acid, palmitoleic acid, on Serine 209 (Takada *et al.*, 2006). This position is well conserved within the Wnt/Wg family, suggesting that other family members might be modified in a similar fashion. Palmitoleic acid incorporation is required for efficient palmitoylation of Wnt3 at Cys 77 (Doubravska *et al.*, 2011; Takada *et al.*, 2006). Modification of Wnt3a with palmitoleic acid regulates secretion, as Wnt3a S209A protein is not recovered in the conditioned media of L cells posttransfection (Takada *et al.*, 2006). As expected given its secretion defect, S209A Wnt3a-RNA injected into *Xenopus* oocytes was unable to cause axis duplication, a hallmark of active Wnt3a signaling (Takada *et al.*, 2006).

Incorporation of palmitoleic acid has yet to be reported for any other member of the Wnt/Wg family. However, the predicted modification site, Ser 239, has been mutated in *Drosophila* Wg (Franch-Marro *et al.*, 2008). Unlike the equivalent mutation in mouse Wnt3a, Wg S239A was readily secreted (Franch-Marro *et al.*, 2008). Moreover, another *Drosophila* Wnt homologue, WntD, lacks the putative palmitoleic acid modification site but is still efficiently secreted from both S2 and L cells, indicating that palmitoleic acid modification is not essential for secretion of all Wnt/Wg family members (Ching *et al.*, 2008). Mutation of Ser 239 does compromise Wg signaling activity, suggesting that palmitoleic acid incorporation may be important for signaling by both mouse Wnt3a and *Drosophila* Wg (Franch-Marro *et al.*, 2008).

In addition to influencing secretion and signaling activity, palmitoylation of Wnt/Wg proteins also affects their extracellular transport. Enhancing palmitoylation of either Wnt1 or Wnt3a in the chick neural tube by ectopic overexpression of the putative palmitoyl transferase, Porcupine, acts to steepen the Wnt signaling gradient, presumably by preventing diffusion away from the site of secretion (Galli *et al.*, 2007). This result suggests that palmitoylation regulates extracelluar transport of Wnt/Wg in a similar fashion to Spitz. However, Wnt family members signal over longer distances. This fact, coupled with the observation that Wg copurifies with apolipophorins has led to the speculation that palmitoylation promotes Wg incorporation into lipoprotein particles (Panakova *et al.*, 2005). Once incorporated into lipoprotein particles Wg would then be able to travel and signal over longer distances.

IV. The MBOAT Family of Acyltransferases

Two groups of protein palmitoyl acyltransferases (PATs) have been identified. The first, which is responsible for palmitoylation of cytoplasmic and membrane bound proteins, is the DHHC family of PATs (Lobo *et al.*, 2002). This family is named for the presence of a conserved Asp-His-His-Cys motif that is required for activity (Lobo *et al.*, 2002). The second family of PATs, the MBOAT family, mediates attachment of palmitate and other fatty acids to lipid and protein substrates. In particular, the MBOAT family members Hhat,

Porcupine, and ghrelin *O*-acyltransferase (GOAT) are responsible for fatty acylation of secreted proteins.

A. MBOAT family characteristics

The MBOAT family of acyltransferases was initially identified by bioinformatics studies. Global sequence alignment algorithms revealed a region of high homology between acyl:CoA cholesterol acyltransferases 1 and 2 (ACAT1/2), the enzymes that catalyze the formation of cholesterol esters, and Porcupine, a segment polarity gene linked to posttranslational modification of Wnt/Wg proteins (Hofmann, 2000). The defining characteristics of the MBOAT homology domain are the presence of a highly conserved His residue surrounded by a cluster of hydrophobic residues and a well-conserved Asp/Asn residue surrounded by moderately hydrophobic residues (Hofmann, 2000). Mutation of one or both of these residues compromise enzymatic activity in all family members tested, suggesting that they may be directly involved in catalysis (Bosson *et al.*, 2006; Chamoun *et al.*, 2001; Guo *et al.*, 2005; Lin *et al.*, 2003; Shindou *et al.*, 2009; Yang *et al.*, 2008a).

All members of the MBOAT family are predicted to be multipass membrane proteins containing between 8 and 12 TM segments. Unlike the DHHC family, most MBOAT family members do not function as protein PATs, but rather catalyze transfer of long chain fatty acids to hydroxyl groups of other lipophilic molecules including cholesterol, diacylglycerol, and lysophospholipids (Cases *et al.*, 1998; Chang *et al.*, 1998; Stahl *et al.*, 2008). Members of the MBOAT family are also involved in processing glycosylphosphatidylinositol anchors and the formation of wax esters (Bosson *et al.*, 2006; Yen *et al.*, 2005). In mammalian cells, MBOAT family members with known substrates can be subdivided into three subfamilies based upon substrate preference and/or amino acid homology. These are: (1) the ACAT family, consisting of ACAT1, ACAT2, and DGAT1 (acyl:CoA diacylglycerol-acyltransferase 1); (2) the LPLAT (lysophospholipid acyltransferases) family, consisting of MBOAT1, MBOAT2, MBOAT5, MBOAT7; and (3) the PAT family consisting of Porcupine, Hhat, MBOA-4 (GOAT), and the mammalian homologue of glycerol uptake protein-1 (GUP1), also known as Hhat-like (Hhatl).

B. MBOAT family members that acylate protein substrates

Three members of the MBOAT family have been implicated in the fatty acylation of secreted proteins (Table 10.1). Hhat/Rasp are the palmitoyl acyltransferases for Hh proteins in flies and mammalian cells, and for Spitz in flies (Chamoun *et al.*, 2001). Porcupine has been implicated in Wnt/Wg palmitoylation (Zhai *et al.*, 2004). GOAT mediates incorporation of an octanoyl moiety into the appetite stimulating peptide hormone ghrelin (Gutierrez *et al.*, 2008; Yang *et al.*, 2008a). Biochemical analyses of these proteins are limited primarily due their hydrophobic nature and the difficulty in establishing *in vitro* assays to monitor fatty acid transfer to substrate proteins.

1. Hhat—Hhat is an ~50kDa multipass membrane protein that, as described in Section II.C, catalyzes attachment of palmitate to hedgehog proteins. Structure–function analysis of Hhat has begun to define regions and residues that are important for Shh palmitoylation. Transmembrane topology mapping predicts at least eight TM domains in Hhat. Truncation

mutants that lack one or two TM segments from either the N- or C-terminal ends of Hhat exhibit reduced stability when expressed in Hhat-transfected cells and are inactive in Shh palmitoylation assays (Buglino and Resh, 2010). Two large loop regions are predicted to be positioned between residues 153–235 and 311–426 in Hhat. Both of these loops contain residues that are highly conserved among MBOAT family members that acylate protein substrates. Deletions or point mutations within these loops reduce Hhat activity without compromising protein stability (Buglino and Resh, 2010). In particular, mutation of the invariant histidine (His379) that is conserved in all MBOAT proteins, reduces palmitoylation activity of Hhat toward Shh by twofold. Kinetic analysis reveals that this mutant binds and transfers palmitoyl CoA as well as the wild-type enzyme, but has an increased K_m and decreased V_{max} for Shh.

2. Porcupine—Several lines of genetic data suggest that Porcupine mediates modification of Wnt/Wg proteins with both palmitate and palmitoleic acid (Kadowaki *et al.*, 1996; Takada *et al.*, 2006; Willert *et al.*, 2003; Zhai *et al.*, 2004). The *porcupine* gene was first identified in genetic screens aimed at identifying mutations that affect segment number and polarity during early *Drosophila* development (Nusslein-Volhard and Wieschaus, 1980). Further analysis of the *porcupine* mutant phenotype indicated that loss of *porcupine* function impaired Wg signaling in *Drosophila* (Kadowaki *et al.*, 1996). Subsequent cloning and characterization of the gene responsible for the *porcupine* phenotype revealed that it encodes a multipass membrane protein that influences Wg processing during transit through the secretory pathway (Tanaka *et al.*, 2002). Loss of Porcupine function reduces the hydrophobic character of Wg as judged by reversed phase chromatography and detergent phase separation (Zhai *et al.*, 2004). In addition, the proportion of Wg present in lipid rafts is reduced when Porcupine function is impaired (Zhai *et al.*, 2004). In mammalian cells, mutation of Porcupine causes a similar reduction in the amount Wnt-3a recovered in the detergent phase after Triton X-114 extraction (Willert *et al.*, 2003). siRNA mediated knockdown of Porcupine inhibits Wnt-3a signaling, indicating that Porcupine is required for Wnt-3a palmitoylation *in vivo* (Galli *et al.*, 2007). However, direct Porcupine mediated lipophilic modification of Wnt/Wg family member at either site has not yet been observed. Loss of Porcupine function has also been observed to result in alteration in the glycosylation status of *Drosophila* Wg, but it is not clear if these effects are direct or indirect (Kadowaki *et al.*, 1996; Tanaka *et al.*, 2002).

The C-terminal end of Porcupine has been implicated in Wg binding. C-terminal truncation mutants of Porcupine fail to Co-IP Wg to the same extent as full-length Porcupine (Tanaka *et al.*, 2002). Apart from this region, no other residues or regions of Porcupine have yet been implicated in the regulation of substrate binding or catalysis.

3. Ghrelin *O*-acyltransferase—Ghrelin is a small peptide hormone that stimulates growth hormone release from the pituitary and also acts to stimulate appetite (Cummings, 2006; Kamegai *et al.*, 2001; Kojima *et al.*, 1999). Mature ghrelin is modified by an O-linked molecule of octanoic acid on Ser-3, which is essential for all known functions (Kojima *et al.*, 1999). Loss of ghrelin by genetic knockout results in a significant reduction in obesity in mice when challenged with a high-fat diet (Kamegai *et al.*, 2001). This observation has led

to increased interest in the regulation of ghrelin octanoylation as a potential drug target for prevention of obesity in humans (Cummings, 2006).

Recently, two independent groups identified the enzyme that catalyzes ghrelin octanoylation, GOAT, for ghrelin *O*-acyltransferase (Gutierrez *et al.*, 2008; Yang *et al.*, 2008a). Overexpression of GOAT stimulates ghrelin octanoylation and mice lacking GOAT are devoid of octanoylated ghrelin in circulating blood (Gutierrez *et al.*, 2008; Yang *et al.*, 2008a). These findings indicate that GOAT is the sole mediator of ghrelin octanoylation *in vivo*. GOAT activity requires the MBOAT homology Asn and His residues, as mutation of either position eliminates GOAT activity both in intact cells as well as *in vitro* (Yang *et al.*, 2008a,b). Thus far no other regions of GOAT have been implicated in catalysis or substrate binding. However, GOAT mediated octanoylation of ghrelin is influenced by the residues that surround the modification site Ser. Mutation of the Gly at position-1 or the Phe at position-4 results in almost complete inhibition of ghrelin octanoylation (Yang *et al.*, 2008b). These results indicate that the amino-terminal residues of ghrelin serve as a GOAT recognition sites (Ohgusu *et al.*, 2009; Yang *et al.*, 2008b). Further studies have shown that peptides derived from the N-terminus of ghrelin are able to inhibit the octanoylation of full-length ghrelin. In these studies, octanoylated peptides were two to four times as potent as nonoctanoylated peptides suggesting that GOAT is subject to product inhibition (Yang *et al.*, 2008b).

V. Conclusions and Future Directions

Palmitoylation has traditionally been viewed as a mechanism to enhance membrane binding of intracellular proteins. It is now clear that secreted proteins, such as Hh, Shh, and Wnt proteins, can be palmitoylated, and that palmitoylation regulates the signaling range and activity of these morphogens in the extracellular space. Hh/Shh palmitoylation is catalyzed by Hhat, is independent of autocleavage and cholesterol attachment, and occurs during passage of Hh/Shh through the secretory pathway. Hedgehog proteins enter secretory organelles by virtue of their signal sequence, but a mechanism for transporting the other substrate, palmitoyl CoA, into the lumen of the ER and/or Golgi must exist. The active site of Hhat is likely exposed to the ER lumen but details regarding Hhat TM topology remain to be determined. To date, known Hhat substrates include Hedgehog proteins and Spitz, but it is likely that other protein substrates for Hhat exist in flies and mammals. Similarities and differences in how two MBOAT proteins, Hhat and Porcupine, recognize their respective substrates, need to be delineated. We also need a better understanding of how lipidation regulates extracellular gradient formation by palmitoylated morphogens. Finally, identification of activators and inhibitors of Hhat as well as other MBOAT proteins will be useful. A more complete understanding of Hhat enzymology could potentially provide avenues for therapeutic intervention in diseases that require ligand dependent activation of the Hh-signaling cascade for growth and survival.

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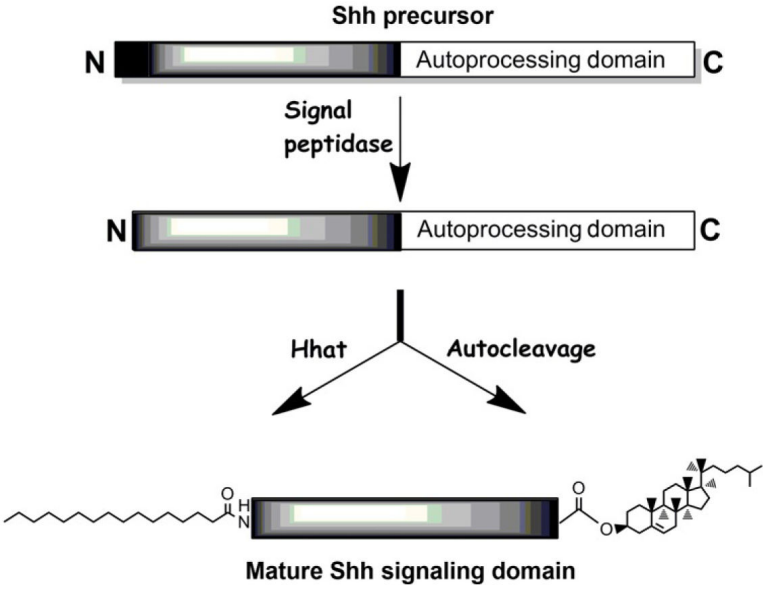

Figure 10.1.

Multiple processing events generate the mature Shh signaling protein. Signal peptidase removes the N-terminal signal sequence. The autoprocessing domain mediates autocleavage and attachment of cholesterol to the C-terminus of the Shh signaling domain, while Hhat catalyzes attachment of palmitate via amide linkage to the N-terminus of the Shh signaling domain.

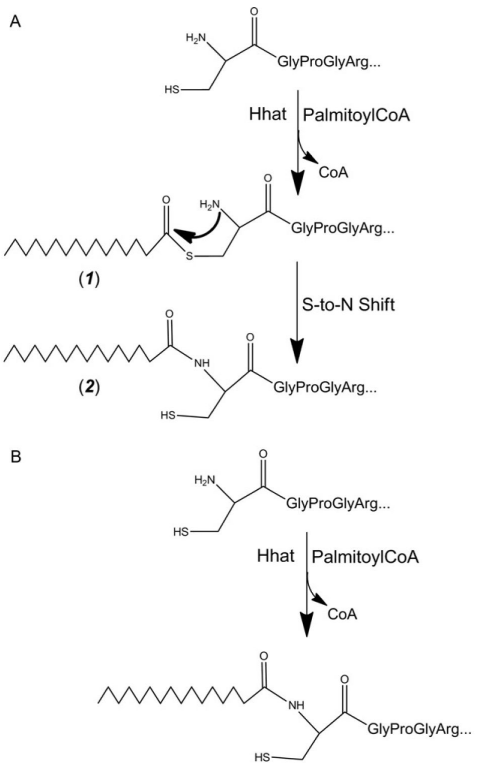

Figure 10.2.

Potential mechanisms for Hhat-mediated *N*-Palmitoylation of Hedgehog proteins. (A) Formation of a thioester intermediate (1) is followed by an S-to-N intramolecular shift and generation of the amide-linked, palmitoylated hedgehog protein (2). (B) Alternatively, Hhat could catalyze direct attachment of palmitate via amide bond to the amine group of the N-terminal cysteine.

Table 10.1

MBOAT family members that catalyze attachment of fatty acids to proteins

Enzyme	Protein substrate	Fatty acid substrate
Hhat	Shh, Ihh, Dhh	Palmitate (16:0)
Rasp	Hh, Spitz, Keren, Gerken	Palmitate (16:0)
Porcupine	Wg and Wnt proteins	Palmitate (16:0) Palmitoleic (16:1)
GOAT	Ghrelin	Octanoate (8:0)