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Fatty Acylation of Proteins: The Long and the Short of it

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Abstract

Long, short and medium chain fatty acids are covalently attached to hundreds of proteins. Each fatty acid confers distinct biochemical properties, enabling fatty acylation to regulate intracellular trafficking, subcellular localization, protein-protein and protein-lipid interactions. Myristate and palmitate represent the most common fatty acid modifying groups. New insights into how fatty acylation reactions are catalyzed, and how fatty acylation regulates protein structure and function continue to emerge. Myristate is typically linked to an N-terminal glycine, but recent studies reveal that lysines can also be myristoylated. Enzymes that remove N-terminal myristoyl-glycine or myristate from lysines have now been identified. DHHC proteins catalyze S-palmitoylation, but the mechanisms that regulate substrate recognition by individual DHHC family members remain to be determined. New studies continue to reveal thioesterases that remove palmitate from S-acylated proteins. Another area of rapid expansion is fatty acylation of the secreted proteins Hedgehog, Wnt and Ghrelin, by Hhat, Porcupine and GOAT, respectively. Understanding how these membrane bound O-acyl transferases recognize their protein and fatty acyl CoA substrates is an active area of investigation, and is punctuated by the finding that these enzymes are potential drug targets in human diseases.

Keywords

myristoylation; myristoyl switch; palmitoylation; depalmitoylation; lipid rafts; MBOAT family

1. Introduction

Proteins can be modified with fatty acids that range in length from eight to over twenty carbons (Table 1). The predominant species acylated to proteins are saturated chain fatty acids, but monounsaturated and polyunsaturated fatty acids can also be attached. There are three major classes of fatty acylation reactions in mammalian cells: N-myristoylation catalyzed by N-myristoyl transferase, S-palmitoylation/acylation catalyzed by DHHC family enzymes, and fatty acylation of secreted proteins catalyzed by MBOAT family enzymes. Each of these fatty acylation reactions utilizes different enzymes, different fatty acyl CoA

and protein substrates, and occurs in different intracellular locations. Advances in structural biology analysis have revealed that, besides enhancing membrane binding, fatty acids can form inter- and intramolecular protein-protein interactions by insertion into a hydrophobic binding pocket.

The 14-carbon, saturated fatty acid myristate is typically linked to an N-terminal glycine via a stable, amide bond. The acylation reaction occurs cotranslationally in the cytosol. Myristate, along with a second signal (polybasic domain, palmitoylation) can promote membrane binding. Although myristate is generally not physically removed, myristoyl switch mechanisms induce regulated release of the modified protein from membranes, by intramolecular sequestration of the fatty acid. Most palmitoylated proteins contain palmitate linked to the sulfhydryl group of one or more cysteines. These posttranslational reactions are catalyzed by the DHHC family of palmitoyl acyltransferases located on the cytosolic side of intracellular membranes or the plasma membrane. The thioester bond is reversible by thioesterases, allowing some palmitoylated proteins to undergo regulated membrane binding and release. Although the vast majority of fatty acylated proteins are intracellular or transmembrane proteins, fatty acids can also be attached to secreted proteins. The MBOAT (membrane bound O-acyl transferase) family catalyzes attachment of palmitate to hedgehog proteins, palmitoleoylate to Wnt proteins, and octanoate to ghrelin. Each of these protein substrates employs a signal sequence to enter the lumen of the endoplasmic reticulum (ER), where they are then fatty acylated. This review will highlight the similarities and differences among these three types of lipidation reactions.

2. N-Myristoylation

2.1 N-myristoyl transferase (NMT) catalyzes N-myristoylation

Covalent attachment of the 14-carbon fatty acid myristate to an N-terminal glycine has been reported for over 150 proteins in mammalian cells [1]. Nearly all N-myristoylated proteins that are co-translationally modified contain the N-terminal consensus sequence: Met-Gly-X-X-X-Ser/Thr. First, the initiating methionine is removed by methionine aminopeptidase. This reaction is essential to expose Gly as the N-terminal amino acid. Next, Myristoyl-CoA:protein N-myristoyl transferase (NMT) catalyzes transfer of myristate from the myristoyl-CoA donor onto the N-terminal glycine via an amide bond. Glycine is the only residue that can serve as an acceptor substrate for NMT, and Gly-to-Ala (G2A) mutations are typically used to generate non-myristoylated protein mutants. Programs with algorithms for prediction of N-myristoylation sites within proteins are listed in Table 2.

NMT is expressed as a single gene in yeast, flies and worms, where it is required for viability [2]. In vertebrates, two genes, *Nmt1* and *Nmt2*, encode NMT proteins with ~75% sequence identity [3]. NMT1 is essential for development, as knockout of *Nmt1* in mice is embryonic lethal [4]. The two isoforms share similar but not identical substrate specificities, and RNAi knockdown experiments reveal functional differences between NMT1 and NMT2 when depleted in cells. For example, depletion of NMT1 reduces cell proliferation, while depletion of both NMT1 and NMT2 with RNAi causes apoptosis [5].

The NMT catalytic reaction proceeds via ordered Bi Bi kinetics [6]. Myristoyl-CoA binds first, with the hydrocarbon chain inserting in a bent conformation into a hydrophobic binding pocket within the enzyme. The size of the binding pocket is only deep enough to accommodate 14 carbons, essentially conferring specificity for myristate over longer fatty acids [7, 8]. Binding of myristoyl-CoA results in formation of a peptide binding site, allowing the protein substrate to bind and form a ternary complex. Myristate is then transferred to the N-terminal glycine, followed by sequential release of CoA and finally the N-myristoylated protein.

Palmitate is too long to fit into the fatty acid binding pocket of NMT. As such, it cannot make the contacts needed for catalysis and thus cannot be transferred to proteins [7]. However, palmitoyl CoA can bind to NMT and competitively inhibit the enzyme *in vitro*. Given that palmitate and palmitoyl-CoA are far more abundant in cells than myristate and myristoyl-CoA, a mechanism must exist to "protect" NMT from inhibition by palmitoyl-CoA. Most of the intracellular fatty acyl-CoA is bound to Acyl-CoA binding protein (ACBP), a 10 kD cytosolic protein that preferentially binds fatty acyl-CoAs between 14–22 carbons with nM affinity[9]. Binding of long chain fatty acyl-CoAs to membranes further reduces the concentration of "free" fatty acyl-CoA. It is possible that, due to its higher affinity for lipid bilayers[10], binding of palmitoyl CoA to cellular membranes segregates this lipid species away from a pool of cytosolic myristoyl CoA that could serve as a substrate for NMT. However, there is an additional consideration. The K_m of NMT for myristoyl CoA is 0.6 μM[11], far above the estimates of free intracellular fatty acyl-CoA concentrations, which range from 5–200 nM[12]. How is sufficient myristoyl CoA delivered to NMT so that it can perform catalysis efficiently? One possibility is that ACBP "donates" the fatty acid as a myristoyl-CoA/ACBP complex to the enzyme. This type of delivery system has been shown to operate for enzymes involved in β-oxidation and glycerolipid synthesis[13] . Another option is illustrated by the recent finding that NMT2 can bind to an Acyl-CoA binding domain containing protein, ACBD6, and this interaction prevents binding of palmitoyl-CoA to NMT2[14]. It has yet to be determined whether this mechanism, which has been demonstrated *in vitro*, is operative in cells.

Although the majority of N-myristoylated proteins undergo fatty acylation during protein translation, a second class of NMT substrates that are N-myristoylated post-translationally has been uncovered[15, 16]. During apoptosis, caspase-mediated cleavage between Asp-Gly exposes glycine at the N-terminus of the newly cleaved product. If this glycine is contained within a myristoylation consensus sequence, the cleavage product can be N-myristoylated. Examples of caspase-cleaved proteins that are post-translationally N-myristoylated include PAK2, gelsolin, actin, and BID[17, 18] and global profiling reveals that there could be as many as 40 such proteins [19]. Some of the newly myristoylated cleavage products are targeted to mitochondrial or other internal membranes, where they regulate apoptosis[17, 18].

2.2 Role of myristoylation in membrane binding

The majority of N-myristoylated proteins are membrane bound. It was initially presumed that attachment of myristate is a *de facto* signal to direct the modified protein to bind to

membranes through hydrophobic interactions. However, this assumption is not correct. In a classic report, Peitzsch and McLaughlin showed that the ΔG_o for binding of a model myristoylated peptide to a phospholipid bilayer is 8 kcal/mol, which corresponds to an effective K_d of 10^{-4} M [10]. This is not sufficient energy to anchor a myristoylated protein stably to a lipid bilayer. In order to achieve stable membrane binding, N-myristoylated proteins employ a second signal[20]. At least four types of second signals have been identified: polybasic regions, hydrophobic residues, another membrane-bound binding partner, or a second lipid modification (Figure 1).

N-myristoylated proteins such as c-Src, MARCKS (myristoylated alanine-rich C Kinase substrate), hisactophilin, and HIV-1 Gag and Nef contain a polybasic region which synergizes with myristate to provide strong membrane association[21–23]. The positively charged residues form electrostatic interactions with negatively charged phospholipids (phosphatidylserine or phosphatidylinositolphosphates) which are enriched on the cytoplasmic leaflet of the plasma membrane and many intracellular organelle membranes. In the absence of myristate, the binding energy from these electrostatic interactions alone is not sufficient to anchor a protein to a membrane. However, when a myristate + basic motif is present within a protein, hydrophobic and electrostatic forces synergize, resulting in strong membrane binding affinity. Myristoylated proteins that lack an obvious polybasic motif can instead use the presence of nearby hydrophobic amino acids as a second signal. For example, hydrophobic residues (Phe, Leu) from the N-terminal helix provide a hydrophobic face to enhance membrane binding of the small G protein ARF1[24]. An alternative mechanism is to utilize protein-protein interactions with a membrane bound binding partner. For example, the binding of N-myristoylated GRASP65 to Golgi membranes is enhanced by interactions with its Golgi-localized receptor GM130[25].

The second signal for membrane binding of an N-myristoylated protein can also be an additional lipid modification. Src family kinases, α subunits of heterotrimeric G proteins, AKAPs (A-kinase anchoring proteins), and endothelial nitric oxide synthase are examples of proteins that contain N-terminal myristate and thioester linked palmitate attached to one or more nearby cysteine residues[26–28] . N-myristoylation occurs first, and provides increased access to membrane-bound palmitoyl acyltransferases. The additional hydrophobicity provided by two or more fatty acids is sufficient to anchor dually fatty acylated proteins to membrane bilayers.

2.3 Reversible membrane binding: cytosolic states and myristoyl switches

The linkage between myristate and the protein is relatively stable, with a half-life essentially equivalent to that of the peptide bond. There are no known de-myristoylases that cleave myristate from the N-terminal myristoyl-glycine. However, the microbial pathogen *Shigella flexneri* expresses a cysteine protease, IpaJ, that removes myristoyl-glycine from the N-termini of host N-myristoylated proteins[29]. Although IpaJ is able to remove myristoyl-glycine from a majority of N-myristoylated proteins *in vitro*, it exhibits apparent specificity for ARF proteins and members of the related ARL (ARF-like) family in *Shigella* infected cells[30]. Specificity is achieved by binding to the GTPase domain of GTP-bound ARFs localized to the Golgi. This explains why Golgi-associated ARF1 is the major substrate for

IpaJ in cells, and provides a molecular explanation for the dramatic alteration in Golgi structure and disruption of the host secretory pathway.

A more generalized mechanism for dissociating N-myristoylated proteins from the membrane is via a myristoyl switch (Figure 1). In this case, the protein exists in two different conformations: a membrane-bound state where the N-terminal myristate is exposed on the surface of the protein and able to promote membrane binding, or a cytosolic state where the fatty acid is buried within a hydrophobic pocket inside the protein. There are multiple ways to switch an N-myristoylated protein from one form to the other. For example, binding of ligand (Ca++ binding to recoverin, GTP binding to ARF1) triggers a myristoyl switch, resulting in reversible association and dissociation from the membrane[31, 32]. A variation on this theme is the myristoyl electrostatic switch which operates for proteins that use a myristate + basic domain for membrane binding. Phosphorylation within the polybasic region by protein kinase C reduces the electrostatic contribution of this domain and releases proteins such as MARCKS and K-Ras4B from the membrane both *in vitro* and in live cells [33–35]. Another example is the myristoyl entropic switch that operates for the HIV-1 Gag protein[36, 37] . The myristoylated N-terminal matrix (MA) domain of HIV-1 Gag can exist in two states, with myristate either sequestered or exposed. Multimerization drives an entropic switch that promotes myristate exposure and membrane binding. Upon infection of a target cell, the Gag precursor is cleaved, MA shifts to a monomeric form, myristate flips inside, and MA is released from the membrane.

A myristoyl-phosphotyrosine switch regulates the kinase activity of the tyrosine kinase c-Abl. Myristate is bound within a hydrophobic cleft of c-Abl, which holds the kinase in an inactive conformation[38]. Gating by the c-Abl SH2 domain allows the protein to undergo a myristoyl-phosphotyrosine switch, whereupon myristate extrusion leads to kinase activation. Another N-myristoylated tyrosine kinase, c-Src, does not undergo a myristoyl switch. However, N-myristoylation does regulate c-Src kinase activity, but by a different mechanism. The presence of myristate has a positive effect on c-Src tyrosine kinase activity. Wild type N-myristoylated c-Src exhibits increased kinase activity, but is targeted for ubiquitination and has reduced intracellular stability compared to a non-myristoylated c-Src mutant[39]. Thus, in addition to regulating membrane binding, myristoylation regulates steady state levels and kinase activity of c-Src. This prevents activated forms of this tyrosine kinase, which could cause malignant transformation, from building up within the cell.

The myristate + basic motif in some N-myristoylated proteins can confer lipid-binding specificity, promoting binding of these proteins to specific membranes. For example, the HIV-1 MA domain binds specifically to phosphatidylinositol 4,5-bisphosphate (PI(4,5)P(2)). This interaction triggers myristate exposure and promotes targeting to PI(4,5)P(2) enriched regions on the cytosolic face of the plasma membrane[37]. The N-myristoylated, calmodulin binding protein known as NAP-22/CAP-23/BASP1 contains a cluster of N-terminal polybasic residues which function as a nuclear localization sequence. BASP1 specifically binds PI(4,5)P(2) in the nucleus. This leads to binding of the BASP1/ PI(4,5)P(2) complex to the promoter regions of Wilms tumor 1 target genes, recruitment of the histone deacetylase HDAC1, and results in repression of transcription[40].

3. Lysine Acylation: Myristoylation and Demyristoylation

Attachment of myristate to the epsilon amino group of lysine was first reported over 20 years ago, when the membrane-bound precursors of the cytokines interleukin 1α and TNFα were both shown to be myristoylated on two internal lysine residues[41, 42]. More recently, interest has been reignited in lysine myristoylation by the finding that lysines on histone proteins, which are traditionally viewed as being acetylated or methylated, can also be modified with a variety of short and long chain acyl groups. These include propyl, butyryl, and crotonyl as well as myristoyl and palmitoyl moieties[43–46].

The enzyme(s) responsible for lysine acylation has not yet been identified. However, a family of enzymes that removes fatty acyl groups from modified lysines has emerged. Sirtuins were originally characterized as a family of 7 NAD-dependent deacetylases which act to remove acetyl groups from acetylated histones. However, *in vitro* assays revealed that deacetylase activity of several of the family members is quite weak. Lin and colleagues demonstrated that Sirt2 and Sirt6 actually have deacylase activity, and preferentially hydrolyze long chain fatty acyl groups (myristoyl, palmitoyl) from histone peptides containing acylated lysine[47] . Co-crystallization in complex with a histone H3K9 myristoylated peptide revealed that myristate binds within a large hydrophobic pocket present within both Sirt2 and Sirt6[47, 48].

The physiologic significance of lysine myristoylation/demyristoylation is starting to be understood. Processing of TNFα involves demyristoylation by Sirt6, an event that is required for efficient secretion of the mature form of TNFalpha into the media[47]. For sirtuins, *in vitro* studies demonstrate reciprocal regulation of deacetylation and deacylation: addition of free fatty acids stimulates deacetylation activity of Sirt6[49] . This suggests that the metabolic state of the cell could regulate histone modification status, ie increased levels of fatty acids would induce histone deacetylation and repression of gene expression.

It remains to be determined whether there are other fatty acylated substrates for sirtuins. Sirt 6, as well as Sirt1 and 7 are localized in the nucleus, whereas Sirt2 is the only sirtuin localized to the cytoplasm. RNAi knockdown or inhibition of Sirt2 was shown to increase the levels of fatty acylation of multiple cellular proteins as well as core histones H1, H2A,H2B, H3 and H4 [50]. Identification of proteins whose lysine fatty acylation levels are altered by sirtuin expression may reveal novel modes of regulation.

4. Protein Palmitoylation

4.1 S-palmitoylation and the palmitoylome

Attachment of the 16-carbon saturated fatty acid palmitate to one or more cysteines via thioester linkage is referred to as S-palmitoylation[26]. The intracellular fatty acid donor is palmitoyl CoA. Several hundred proteins have been reported to be S-palmitoylated. These encompass a wide range of functions, including signaling proteins, receptors, ion channels, and transcriptional regulators. The modified cysteines can be located near the N- or C-terminus, or within internal regions of the protein. Chemical biology approaches combined with mass spectrometry analysis has led to cataloging of “palmitoylomes” in a variety of cell

and tissue types[43, 51–55]. Although there is no overall consensus sequence that distinctly characterizes all known S-palmitoylated proteins, algorithms have been developed to predict potential palmitoylation sites (Table 2)[56]. A recent compilation drawn from over 5,000 S-palmitoylated proteins suggests that as much as 10% of the human proteome consists of S-palmitoylated proteins[57].

4.2 DHHC enzymes catalyze S-palmitoylation

S-palmitoylation of proteins is catalyzed by DHHC palmitoyl acyltransferases[58, 59] . Each member of this family of enzymes (5–7 in yeast, 23 in humans) contains a highly conserved Asp-His-His-Cys sequence within a cysteine-rich domain. DHHC proteins are predicted to have four to six transmembrane domains. These enzymes are localized at the plasma membrane or in intracellular membranes (ER, Golgi, endosomes), with the active site exposed to the cytoplasmic side of the membrane. In addition to the 50 amino acid DHHC homology domain, DHHC acyltransferases also contain other domains involved in protein-protein interactions, such as PDZ domains and ankyrin repeats. This may provide a docking function to promote substrate selection. There is a fair amount of functional redundancy built into the mammalian enzyme system: many palmitoylated proteins can be modified by more than one DHHC family member and each DHHC enzyme can apparently palmitoylate more than one protein substrate[60–62]. Moreover, fatty acids other than palmitate can be S-acylated to proteins (see Section 4.3), and evidence for selectivity in fatty acid transfer has been obtained for DHHC2 and DHHC3[63]. Studies of mice with loss of function alleles or knockout of a single DHHC gene have uncovered distinct phenotypes [61]. For example, mice deficient in DHHC13 or DHHC17 exhibit neurological defects similar to Huntington disease [64, 65], while defects in vascular tone and hair follicle differentiation result when mice produce non-functional DHHC21 [66, 67].

DHHC catalyzed S-palmitoylation follows a two step ping-pong enzymatic mechanism[63, 68] . First, palmitate is attached to the cysteine in the DHHC motif[69]. This autoacylation reaction results in formation of an acyl-enzyme intermediate, which then transfers palmitate to a cysteine residue within the acceptor protein substrate. Structural integrity of DHHC enzymes is maintained by coordination of two zinc ions to conserved cysteines within the cysteine rich domain[69]. Although mutation of the DHHC cysteine residue typically generates an inactive enzyme mutant, there are examples of yeast DHHC proteins that can function at reduced efficiency with alanine or arginine in place of the DHHC cysteine[70].

S-palmitoylation can occur on proteins with another lipid modification[20]. For example, N-myristoylated proteins that contain a cysteine residue located at or near an N-terminal glycine (eg Src family kinases, Gα subunits, eNOS) can be S-palmitoylated to produce a dually fatty acylated protein. Prenylated proteins with a cysteine upstream from the C-terminal CAAX-box can be S-palmitoylated (eg H-Ras, N-Ras, K-Ras4A)[71]. In a variation on a theme, proteins that contain a double cysteine (CCAX) in their C-terminal CAAX box have been shown to undergo tandem prenylation and palmitoylation[72]. In the above examples, prior myristoylation or prenylation is required for palmitoylation to occur. The weak hydrophobicity of the myristate or farnesyl group allows the singly modified protein to sample multiple intracellular membranes. In the absence of palmitate, the protein will cycle

between membrane and cytosol. When a suitable palmitoyl transferase is encountered, the substrate becomes dually lipidated, and the increase in hydrophobicity reduces the kinetic "off" rate. As a result, the dually modified myristoylated and palmitoylated or farnesylated and palmitoylated protein becomes trapped at the membrane where it was modified. This mechanism, known as kinetic bilayer trapping, enables a particular palmitoyl transferase to dictate the subcellular localization of the lipidated protein to a specific membrane[73].

4.3 Heterogeneous S-acylation

In addition to palmitate, mass spectrometry analyses reveal that longer and shorter fatty acids can be attached to "palmitoylation" sites in S-acylated proteins[74, 75]. These include myristate, palmitoleate, stearate, and oleate, and the polyunsaturated fatty acids arachidonate and eicosapentanoate. In some cases, the same cysteine residue can be acylated with multiple fatty acids (eg rabbit sarcolipin is S-acylated with either palmitate or oleate in a 60/40 ratio[76]). For proteins with multiple palmitoylated cysteines, the heterogeneous fatty acylation pattern is different for each cysteine. For example, the hemagglutinin protein (HA) of influenza virus is S-acylated at three cysteines: two contain only palmitate whereas a cysteine residue near the end of the TMD is modified by stearate[77]. The location of the cysteine relative to the TMD was shown to be critical for stearate attachment.

The presence of different fatty acids on a protein can have functional consequences. Altering the fatty acid composition of the growth media, by feeding cells with an excess of a particular fatty acid, was shown to alter the fatty acylation pattern of S-acylated proteins and regulate signal transduction by Src family kinases [74, 78]. Attachment of saturated fatty acids promotes lipid raft association (see section 4.5) and signaling, while acylation with unsaturated fatty acids has the opposite effect . A recent study reported that attachment of stearate to the transferrin receptor, which is known to be S-palmitoylated[79], inhibits its signaling activity and as a consequence, stimulates mitochondrial fusion[80]. These experiments suggest potential therapeutic utility for dietary intervention by supplementation with specific fatty acids.

4.4 Depalmitoylation

S-palmitoylation differs from N-myristoylation in that the thioester link between the fatty acid and the protein chain is reversible. Two acylprotein thioesterases, APT1 and APT2, that cleave palmitate from S-palmitoylated proteins were initially identified. APT1 and APT2 were reported to catalyze depalmitoylation of membrane-bound substrates such as H-Ras and GAP-43. However, it was unclear how these thioesterases, which are cytosolic enzymes, can access their membrane-bound substrates. It was recently shown that APT1 and APT2 are themselves S-palmitoylated, which is required for membrane association and deacylation of H-Ras and GAP-43[81]. APT1 can also depalmitoylate itself as well as APT2, promoting membrane to cytosol shuttling. An alternative view proposes that the soluble, non-palmitoylated forms of APT are responsible for depalmitoylation of membrane-bound substrates and that this serves to remove non-specifically adsorbed Ras proteins from all internal membranes and enable them to be concentrated on the plasma membrane[82].

APT1 and APT2 are members of a serine hydrolase superfamily which, until recently, had been presumed to be solely responsible for depalmitoylation of all intracellular S-palmitoylated proteins. However, knockdown or inhibition of APT1/APT2 did not alter turnover of palmitate on N-Ras or PSD-95. This finding led to the identification of ABDH17 proteins (isoforms A,B,C), palmitoylated serine hydrolase family members that are capable of catalyzing depalmitoylation of N-Ras and PSD95 and altering palmitate turnover on N-Ras[83]. Using a lipid hydrolase inhibitor and activity based protein profiling, an additional 20 members of the serine hydrolase family have also been implicated as potential depalmitoylating enzymes [52].

4.5 Functions of S-palmitoylation

For proteins that lack a transmembrane domain(s), one of the obvious functions of palmitate is to promote membrane binding of the modified protein. Palmitate is sufficiently hydrophobic so that by itself, or in combination with additional lipids, strong association with a lipid bilayer is achieved. In combination with kinetic trapping, as discussed above, palmitoylation provides not only membrane binding but also membrane targeting. Due to the labile nature of the thioester bond, reversible cycles of palmitoylation/depalmitoylation allow this class of lipidated proteins to dynamically cycle between membranes and the cytosol. The best studied examples of reversible membrane binding are H-Ras and N-Ras, small G proteins that are dually lipidated with farnesyl and palmitate[84, 85]. H- and N-Ras are first prenylated by farnesyl transferase in the cytosol. The C-terminal CAAX box is further processed in the ER by removal of the three C-terminal amino acids and C-terminal carboxymethylation. Upon transport to the Golgi, one (N-Ras) or two (H-Ras) cysteines upstream from the C-terminal farnesyl are palmitoylated by DHHC9/GCP16, a DHHC enzyme complex localized to the Golgi. Vesicles generated from the Golgi are trafficked through the secretory pathway to the plasma membrane, where Ras proteins can be released by depalmitoylation and traffic back to the Golgi by diffusion. The dynamic cycle of acylation/deacylation maintains H-Ras and N-Ras at both the plasma membrane and the Golgi, where they encounter different sets of effector proteins and can activate different signaling pathways[86].

Palmitoylation plays an important role in directing proteins to lipid rafts, membrane subdomains that are highly enriched in cholesterol, glycosphingolipids, and phospholipids containing saturated fatty acid chains. The saturated nature of the fatty acid preferentially drives insertion of palmitoylated proteins into the liquid ordered phase found in raft lipids[74, 78, 87]. For example, Src family tyrosine kinases that are dually acylated with myristate and palmitate are raft associated, whereas substitution of palmitate with mono- or polyunsaturated fatty acids reduces raft localization. For these proteins, which would be soluble in the absence of fatty acylation, palmitate plays a triple role: membrane binding, plasma membrane targeting, and lateral targeting to lipid rafts within the plasma membrane.

There are numerous examples of proteins that contain one or more transmembrane domains (TMDs) that are also S-palmitoylated[26]. The role of palmitate in these cases goes beyond just providing a membrane anchor[88]. For proteins contain TMDs longer than the standard 20 amino acid TMD, hydrophobic mismatch would result from extending a TMD beyond

the boundary of the lipid bilayer. S-palmitoylation can address this energetically unfavorable situation in two ways. One option is to promote partitioning of transmembrane proteins into membrane rafts within the ER, as the liquid ordered domain region of the membrane is thicker than the bulk, disordered membrane region. Budding of raft-enriched transport vesicles has been proposed as a sorting signal to direct proteins such as LAT to the plasma membrane[89]. For proteins that have palmitoylation sites adjacent to TMDs (eg LRP6), palmitate may promote tilting of the transmembrane helix to allow proteins with long TMDs to fit within a thinner ER membrane[88]. In both cases, non-palmitoylated mutants would be retained in the ER and exhibit a mislocalization phenotype.

S-palmitoylation can influence protein structure and function by providing an additional anchoring point to the membrane. For example, palmitate-mediated tethering to the bilayer of a region close to a TMD in the γ subunit of the epithelial sodium channel regulates ion channel activity by stabilizing the open state of the channel[90]. When palmitate is present within a long cytoplasmic region, an additional loop will be formed when palmitate inserts into the bilayer. This has been shown to regulate GPCR activity by altering the receptor's ability to bind to different signaling effectors [91].

S-palmitoylation can also influence protein function by altering the accessibility of this region to other post-translational modifications. There are numerous examples of palmitoylation interfering or competing with phosphorylation or ubiquitination at nearby sites [88, 92]. Moreover, analysis of the postsynaptic density protein PSD-95 reveals that modification of the same cysteine residue can be reciprocally regulated by S-nitrosylation vs S-palmitoylation [93].Another mode of regulation is by direct effects on protein-protein interactions. For example, palmitoylation enhances specific interactions between synaptotagmins or integrins and tetraspanins[94, 95]. Finally, at least two proteins have been identified that contain palmitate buried intramolecularly in a deep hydrophobic pocket within the protein. BET3 is a component of the transport protein particle (TRAPP) tethering complex that regulates recruitment and trafficking of of ER-derived vesicles to and through the Golgi. Bet3 exhibits autopalmitoylation activity, which attaches palmitate to an internal cysteine residue. The fatty acid inserts into a hydrophobic groove, where it is required to maintain protein stability[96, 97]. TEAD transcription factors, together with their co-activators YAP and TAX, regulate the Hippo signaling pathway. When purified from E. coli, TEAD proteins were shown to contain covalently bound palmitate, which was attached as a result of autopalmitoylation activity. Mutation of the palmitoylation site in TEAD has no effect on nuclear envelope localization or membrane binding, but reduces interaction with its coactivators and impairs transcriptional activity[98, 99].

5. Fatty acylation of secreted proteins by MBOAT enzymes

5.1 N-palmitoylation of hedgehog proteins

Studies of the hedgehog family of proteins revealed a new mode of attachment for palmitate, via amide linkage to an N-terminal cysteine[100]. Hedgehog proteins (herein referred to as Hh) are secreted from the producing cell and function as morphogens to signal to surrounding cells in a concentration dependent manner. This signaling protein family (hedgehog in flies, Sonic, Indian and Desert hedgehog in mammals,) plays critical roles in

embryonic patterning during development and tumorigenesis in adults. Hh is synthesized as a 45kDa precursor protein containing an N-terminal signal sequence[101]. Upon entry into the ER, the N-terminal signal sequence is cleaved by signal peptidase (Figure 2). Hh contains an intein-like autoprocessing domain which promotes autocleavage. The C-terminal 25 kDa fragment is degraded in the lumen of the ER. The remaining 19 kDa N-terminal fragment is further modified by attachment of two lipids. Cholesterol is covalently attached to the C-terminus, and this occurs concomitantly with autocleavage. In a separate, distinct reaction, palmitate is attached through an amide bond formed between the fatty acid and the amino group of the N-terminal cysteine. The end result is a dually lipidated, mature signaling protein.

N-palmitoylation of Hh is catalyzed by Hhat (Hedgehog acyltransferase) a 57 kDa membrane protein that primarily resides in the ER[102, 103]. The fatty acylation reaction occurs in the lumen of the ER and requires prior removal of the N-terminal signal sequence. N-palmitoylation is independent of C-terminal cholesteroylation; the reaction can occur on the unprocessed Hh precursor in the absence of autocleavage and cholesterol attachment [102]. Hhat has been purified to homogeneity and shown to function as a bona fide palmitoyl acyltransferase with specificity for hedgehog proteins[102]. The N-terminal region of Hh contains the sequence information necessary for substrate recognition: a 10 amino acid peptide can be palmitoylated by Hhat *in vitro*[102], and fusion of the first 6 amino acids of the mature Hh sequence to GFP is sufficient to confer N-palmitoylation to a GFP fusion protein when expressed in cells[104]. Both Hhat and the fly ortholog Rasp require the presence of a positively charged amino acid within the N-terminal sequence for N-palmitoylation to occur[104].

Deletion of the Hhat gene in mice is embryonic lethal, and recapitulates the phenotype of Shh knockout mice[105] . Similar results were obtained for Hh and Rasp deletion in flies[106, 107]. Notably, a Hh mutant that cannot be palmitoylated cannot rescue the Rasp −/− phenotype. These findings indicate that palmitoylation is required for Hh signaling activity during development. In addition to Hh, the EGF-like ligands Spitz, Keren and Gurken are substrates for N-palmitoylation by Rasp in flies[108]. To date, no mammalian equivalents that are N-palmitoylated have been found. By contrast, cholesterol attachment to Hh helps shape the morphogenetic signaling gradient, but is not absolutely essential for Hh signaling in vivo[109].

The presence of two lipid modifications on a protein leads to the question: how is such a hydrophobic molecule released from the cell? One possibility is that the lipids are removed. Cleavage of both N- and C-terminal residues from Shh by metalloproteases has been observed[110], but it is not clear if this type of “shedding” is universally used. The more likely mechanism is by regulated release (Figure 2). Hh producing cells contain two proteins, Dispatched and Scube2, whose functions are to bind and promote release of lipidated Hh into the surrounding media[111, 112]. In the absence of either protein, Hh remains bound to the cell surface. Dispatched is a plasma membrane-bound protein with a sterol sensing domain that recognizes the aliphatic chain of cholesterol bound to Hh. Scube 2, a secreted protein, recognizes the cholesterol ring structure and its efficiency in promoting Hh release is enhanced by Hh palmitoylation. Once lipidated Hh is released from the cell, it

forms multimers in the extracellular milieu, with the hydrophobic groups facing the inside of a micellar structure[113]. These multimeric complexes are presumed to be the active signaling species, but alternative models where Hh travels with lipoprotein particles or is released from exosomes on filopodia-like extensions termed cytonemes have also been proposed[114–116].

5.2 Wnt proteins are palmitoleoylated

Wnt proteins are modified by a monounsaturated form of palmitate, cis-Δ9-palmitoleate (C16:1$^{\Delta 9}$), which is attached via oxyester linkage to a conserved serine residue (Ser209 in Wnt3a)[117, 118]. Wnts comprise a family of cysteine-rich, secreted glycoproteins that regulate embryonic patterning during development as well as stem cell and tissue renewal in adults. Palmitoleoylation is required for Wnt proteins to bind to Wntless, an intracellular transmembrane protein that promotes Wnt secretion by facilitating Wnt movement from the trans Golgi network to the plasma membrane[119, 120]. In addition, palmitoleate binds within a hydrophobic cleft of the Wnt receptor Frizzled, promoting ligand-receptor interactions[121]. Thus, this monounsaturated palmitate analog is required for Wnt trafficking, secretion, receptor binding and signaling.

The enzyme that catalyzes Wnt protein acylation is Porcupine (Porcn), a multipass membrane protein that is predicted to contain 11 TMDs [117, 122, 123]. The reaction occurs in the lumen of the ER in two steps (Figure 3)[124]. First, stearoyl CoA desaturase (SCD) inserts a *cis*-double bond at fatty acid position 9 of palmitoyl CoA to form palmitoleoyl-CoA. This monounsaturated fatty acyl CoA is the substrate for Porcn, which transfers the 16:1 fatty acid to the recipient cysteine in Wnt proteins. Porcn can attach monounsaturated fatty acids that are shorter, but not longer than 16 carbons to Wnt proteins[124, 125], and recognizes a consensus sequence surrounding the conserved cysteine in Wnt proteins[126, 127].

The presence of a fatty acid on Wnt poses the same problem as it does for Hh: how is Wnt released from the cell and how does it travel and signal at multiple cell distances? Several new insights into these questions have recently been obtained. Wnt proteins can be detected in the conditioned media from cultured cells, and mechanisms including secretion on exosomes or binding to lipoprotein particles or Wnt interacting proteins have been proposed to carry Wnt proteins through the extracellular milieu[128–130]. However, when Wingless, the major Drosophila Wnt protein, was tethered to the membrane by fusion with a transmembrane protein, embryonic patterning still occurred in a manner similar to that mediated by wild type Wingless[131]. This suggests that short range signaling is critical during development. In organoids formed from mouse intestinal cells, Wnt is apparently transferred from the producing cell to a neighboring cell, where it remains on the cell surface bound to Frizzled[132]. A short range Wnt gradient is then propagated and diluted by cell division.

Wnt palmitoyleolation has recently been shown to be reversible. The fatty acid can be cleaved from secreted Wnt by Notum, a secreted Wnt antagonist that is a member of the α/β hydrolase superfamily[133, 134]. The deacylase activity is specific for Wnt proteins, as Hh is not depalmitoylated by Notum. The shape of the binding pocket in Notum restricts

binding to cis-monounsaturated fatty acids, thereby explaining selectivity for Wnt proteins. By removing palmitoleate, which is essential for Wnt binding to Frizzled, deacylation by Notum inactivates Wnt signaling *in vitro* and *in vivo*.

5.3 Ghrelin is modified by octanoate

The eight-carbon fatty acid, octanoate, is covalently attached to ghrelin, a secreted 28 amino acid peptide hormone. Ghrelin is synthesized primarily in the stomach. The mature peptide is generated by proteolytic processing from a preprohormone precursor. Fatty acylation is required to produce the active octanoylated form. Only octanoylated ghrelin can bind to the growth hormone secretagogue type 1a receptor, a G protein coupled receptor. The physiologic outputs include growth hormone secretion, appetite stimulation, and increased food uptake and adiposity. Octanoylation of ghrelin is catalyzed by GOAT (ghrelin O-acyltransferase), a multipass membrane protein localized primarily in the gastrointestinal tract[135, 136]. The fatty acid is linked to serine-3 within the N-terminal GSSFL sequence, with glycine-1 and phenylalanine-4 also contributing to enzyme-substrate recognition[137]. Bioinformatics analyses suggest that ghrelin is the only substrate for GOAT in the human proteome.

5.4 How are Fatty Acyl CoA substrates delivered to and recognized by MBOAT enzymes?

Hhat, Porcn and GOAT are members of the MBOAT (Membrane bound O-acyl transferase) family of multipass membrane proteins[138, 139]. Of the 16 MBOAT family members in humans, most catalyze transfer of fatty acids to lipids; only 3 (Hhat, Porcn, GOAT) transfer fatty acids to proteins. The substrates for these three enzymes are secreted proteins, which enter the lumen of the ER via a signal sequence. The fatty acyl donors are the CoA derivatives. In order for fatty acylation to occur, fatty acyl CoA must also be present in the ER lumen. Fatty acids are esterified to fatty acyl CoA in the cytosol, but long chain acyl-CoA esters are not permeable across biological membranes. Thus, a key unanswered question is: how do the long chain fatty acyl CoA substrates access the lumen of the ER? Mitochondria solve this problem with a carnitine-dependent acylation and transfer system: carnitine palmitoyl-transferase1 (CPT1) generates fatty acylcarnitine from fatty acyl CoAs in the cytosol; acylcarnitine traverses the inner membrane bilayer via a carnitine/acylcarnitine exchange carrier (CAC); CPT2 re-forms fatty acyl CoAs in the organelle interior. Carnitine acyltransferase and acylcarnitine exchange activities have been detected and characterized in microsomal membranes[140] but it is not known whether the microsomal enzymes are splice variants of the mitochondrial enzymes or related proteins. Alternatively, it is possible that MBOAT proteins themselves facilitate fatty acyl CoA transport across the ER membrane. Studies of membrane topology reveal that Hhat contains ten TMDs and two reentrant loops[141, 142], with a putative active site histidine disposed near the lumenal surface. Of note, a large fraction of the Hhat sequence is located on the cytosolic side of the ER membrane. Studies of GOAT TMDs reveal a similar membrane topology[143]. The function of the cytosolically disposed loops of these enzymes is unknown, but it is tempting to speculate that these regions may regulate substrate availability and/or selectivity.

MBOAT family members were defined by their *O*-acyl transferase activity. Porcn and GOAT attach fatty acids via O-acylation to serine in their respective substrates, but threonine has also been shown to serve as an acylation acceptor. At first glance, Hhat is different in that palmitate is attached to cysteine via amide bond. However, in a cell based palmitoylation assay, Shh with an N-terminal serine can also serve as a palmitate acceptor, but at reduced efficiency[104]. This finding may explain why N-terminal cysteine to serine mutants of Shh retain residual signaling activity. Use of a cysteine to alanine mutant is a better choice to mimic fully inactive Shh.

6. Fatty acyl transferases as targets in human diseases

Fatty acylated proteins are critical players in signaling in both normal cells and disease states. Targeted inhibitors that selectively block specific fatty acyl transferases are therefore of great therapeutic interest. The key is to be able to block modification of the protein(s) responsible for the disease state without affecting housekeeping proteins that need to be fatty acylated to maintain normal cellular homeostasis. To date, this approach has been successful for NMT and the MBOAT proteins.

N-myristoylation is necessary for the growth and survival of a number of human pathogens, and many of these organisms encode their own NMTs. Although the myristoyl-CoA binding site is highly conserved between human and pathogen NMTs, the peptide binding sites are quite different. By taking advantage of these differences, NMT has been targeted in *Candida albicans, Trypanosoma brucei, and Leishmania major*, organisms that cause fungal infections, African sleeping sickness, and leishmaniasis, respectively[144–146].

The Hh signaling pathway is highly active during embryonic development, but is largely shut off in adult tissues. However, aberrant overexpression of Shh has been shown to drive proliferation and metastasis in numerous human tumors, including pancreatic, breast and lung cancers. Thus, Hhat has the potential to provide a tumor-specific target. Using high-throughput screening and an Shh palmitoylation assay as a readout, potent, selective Hhat inhibitors have been identified and shown to block the growth of pancreatic adenocarcinoma cells as well as estrogen receptor positive breast cancer cells[147–149].

Wnt driven cancers provide an attractive system for development of Porcn inhibitors. Proof of concept that small molecule inhibitors of Porcn can selectively inhibit Wnt signaling was first reported in 2009[150]. Since then, orally bioavailable Porcn inhibitors have been developed that show efficacy in Wnt-dependent cancers[151, 152]. Two optimized Porcn inhibitors, LGK974 and ETC-159, are currently in Phase I clinical trials for potential treatment of cancers dependent on Wnt ligand.

Weight gain is stimulated by acyl-ghrelin, making GOAT an obvious target for potential control of obesity and obesity-associated disorders. A bisubstrate analog, GO-CoA-Tat, linking octanoyl CoA and an N-terminal ghrelin peptide, was shown to be a selective GOAT inhibitor *in vitro* and *in vivo* and prevented weight gain in mice fed a high fat diet[153]. However, studies in GOAT knockout mice have not mirrored the effects of the GOAT inhibitor. GOAT −/− mice grow at the same rate and achieve the same weight as wild type

cohorts, even on a high fat diet[154, 155]. Of note, GOAT −/− mice exhibit reduced food intake and resistance to obesity when placed on a high-fat, high-sucrose diet[155]. Complex interactions with other signaling axes that regulate appetite may complicate the ultimate *in vivo* use of GOAT inhibitors to control obesity and/or diabetes.

7. Conclusions and perspectives

We have now come to appreciate that protein fatty acylation is not limited to modification by just myristate or palmitate, but encompasses a broad range of saturated and unsaturated fatty acids of varying chain length. We also understand that distinct enzymes are responsible for catalyzing subsets of these fatty acylation reactions. In some cases, there is a dedicated fatty acyltransferase, eg NMT for N-myristoylation, Hhat for Hh, Porcn for Wnts, and GOAT for ghrelin. What we do not understand is why there are so many DHHC enzymes and why there is so much potential redundancy in the S-palmitoylation machinery. We also cannot explain how palmitoylation sites are selected, and how and why palmitate turns over rapidly on some proteins, but not on others. Advances in proteomics have led to identification of even more (potential) fatty acylated proteins, but are we done yet? Are there additional fatty acylated proteins, acyltransferases and/or deacylating enzymes yet to be identified?

Another key concept is that not all fat looks the same to the cell. Myristate and palmitate differ by just two CH_2 groups, but have different hydrophobicities and are recognized as substrates by different enzymes at different times and in different places. Palmitate and palmitoleoylate differ by one double bond, but yet only 16:1 is recognized by Porcn and attached to Wnt proteins. The function of heterogeneous fatty acylation is still not clear. What does stearate do to alter the function of the transferrin receptor that is not accomplished by palmitate? Can dietary intervention be successfully used to alter the palette of fatty acids attached to signaling proteins and thereby affect cell signaling?

MBOAT proteins that catalyze fatty acylation of secreted proteins are attractive drug targets in disease, but there is still much to be learned about these enzymes and their acylated substrates. How is the fatty acyl CoA delivered to the lumen of the ER for presentation to the MBOAT enzyme active site? How is release of the lipidated protein into the extracellular milieu regulated and how do lipidated secreted morphogens travel in the aqueous environment? Continued advances in fatty acylation detection methods as well as live imaging promise to deliver answers to some of these questions.

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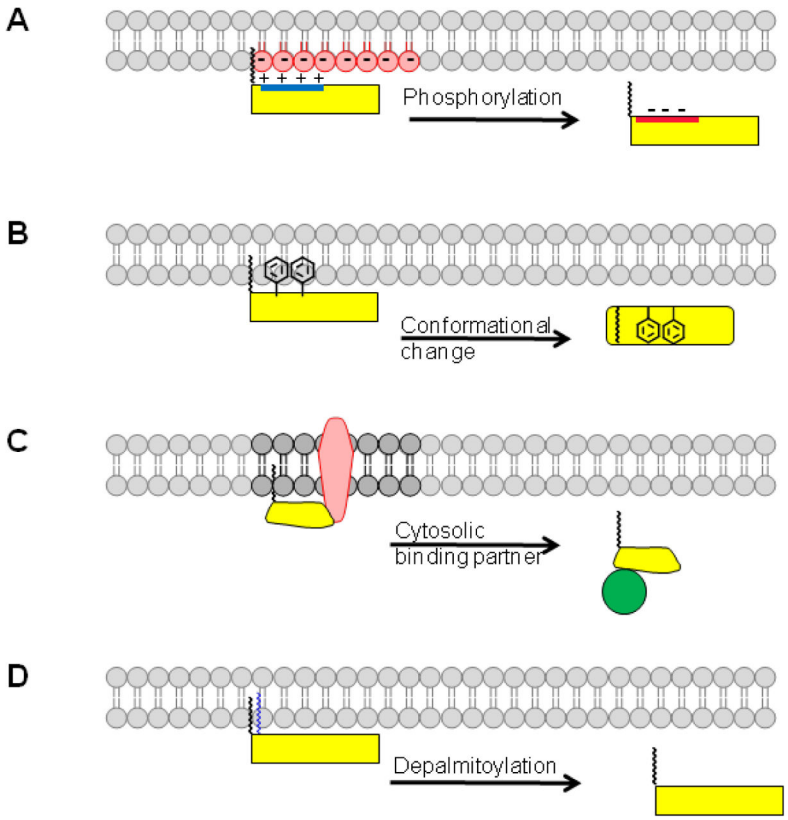


Figure 1. Membrane binding mechanisms for N-myristoylated proteins

Four different scenarios are illustrated where a second signal, in addition to myristate, contributes to membrane binding. (A) A polybasic cluster of amino acids (blue) enhances membrane binding through electrostatic interactions with head groups of negatively charged phospholipids (red). Phosphorylation of residues within the polybasic cluster reduces or neutralizes the positive charge in the protein, resulting in membrane detachment. (B) Hydrophobic amino acids disposed along the membrane proximal surface of the N-myristoylated protein contribute to membrane binding. Conformational changes can lead to sequestration of the myristate (myristoyl switch) and/or reduced surface accessibility of the hydrophobic residues, leading to release of the protein from the membrane. (C) Interaction with another membrane protein can direct N-myristoylated proteins to the membrane. The protein could detach if another binding partner, with higher affinity, interacts with the N-myristoylated protein and releases it into the cytosol. (D) Palmitoylation of an N-myristoylated protein induces membrane binding, which is reversed upon deacylation.

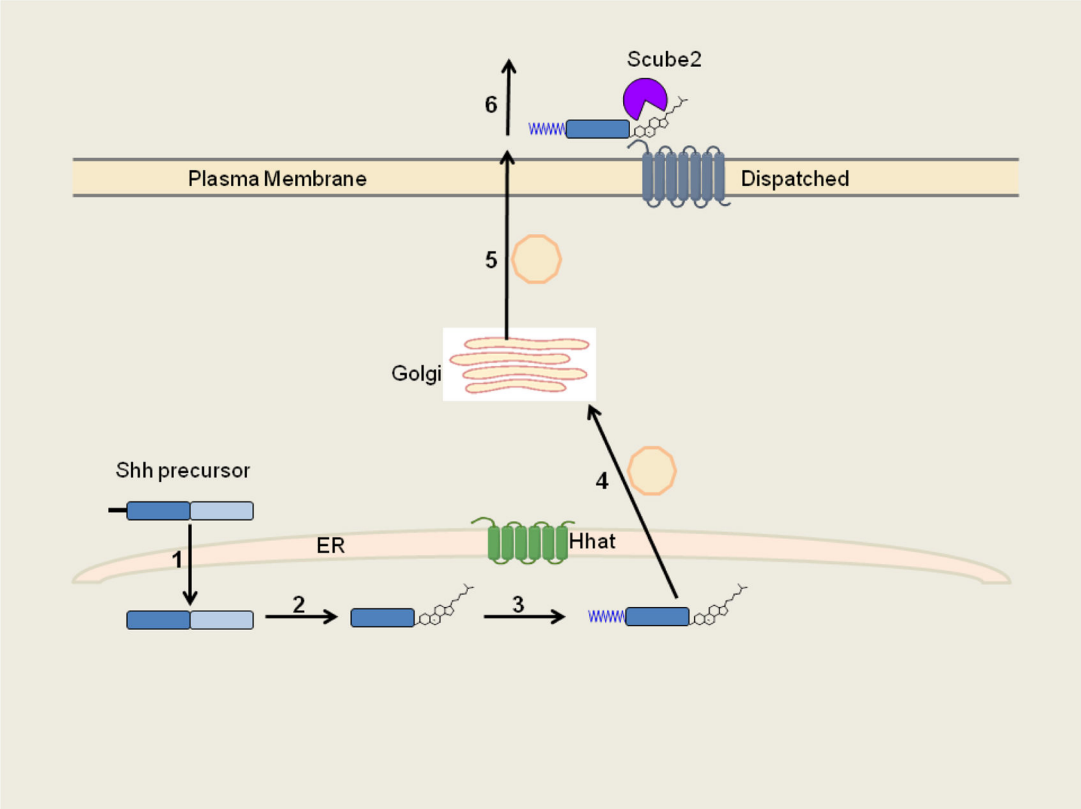


Figure 2. Fatty acylation and secretion of lipidated Sonic Hedgehog

Shh undergoes multiple processing steps: 1) The Shh precursor polypeptide enters the lumen of the ER, where the signal peptide is removed. 2) Autocleavage and cholesterol attachment. 3) Hhat catalyzes palmitoylation of the Shh N-terminus. 4,5) Shh undergoes vesicular trafficking from the ER (step 4) through the Golgi (step 5) and is delivered to the cell surface. 6) Shh release is promoted by interaction with Dispatched and Scube2.

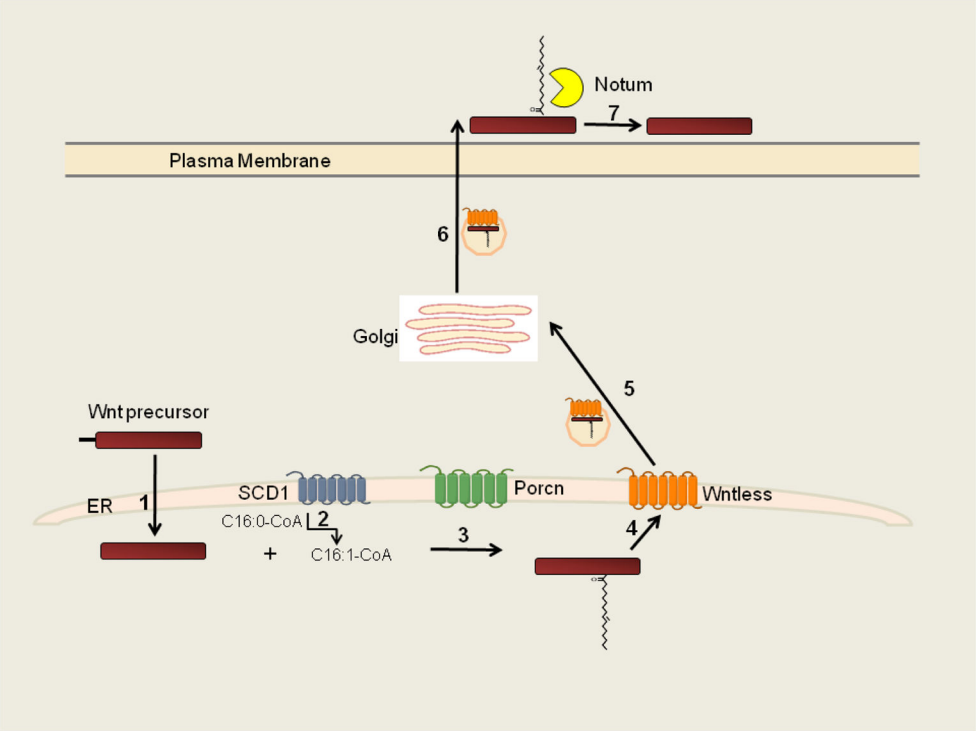


Figure 3. Fatty acylation and secretion of palmitoleoylated Wnt proteins

1) The Wnt precursor enters the lumen of the ER, where the signal peptide is cleaved. 2) SCD1 catalyzes formation of palmitoleoyl-CoA from palmitoyl-CoA. 3) Porcn catalyzes attachment of palmitoleoylate to Wnt. 4) Palmitoleoylated Wnt interacts with Wntless and (5) is packaged into vesicles that transit through the Golgi and then to the plasma membrane (6), where palmitoleoylated Wnt is released. 7) In some cells, Notum releases the fatty acid from Wnt.

Table 1

Biochemistry of protein fatty acylation

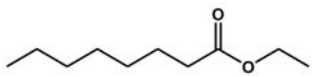
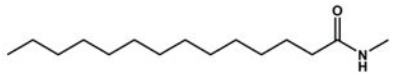
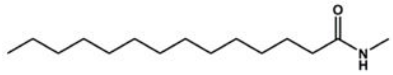
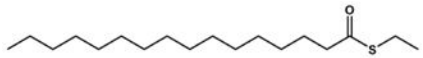
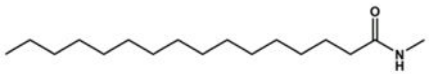
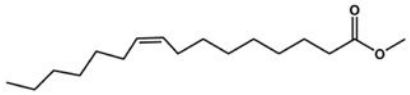
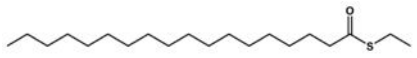
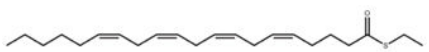
Lipid	Structure	Linkage	Modified Residue	Fatty acyl Transferase	Deacylation?
Octanoate 8:0		Oxyester	Ser	GOAT	unknown
Myristate 14:0		Amide	Gly	NMT	Removal of Myr-Gly by IpaJ
Myristate 14:0		Amide	Lys	unknown	Sirtuins
Palmitate 16:0		Thioester	Cys	DHHCs	Thioesterases (APT1,2; ABDH17)
Palmitate 16:0		Amide	Cys	Hhat	unknown
Palmitoleic 16:1Δ9		Oxyester	Ser	Porcn	Notum
Stearate 18:0		Thioester	Cys	DHHCs?	Thioesterases?
Arach-idonate 20:4		Thioester	Cys	DHHCs?	Thioesterases?

Table 2

Prediction programs for Protein Fatty Acylation

Fatty acid modification	Website
N-myristoylation	http://mendel.imp.ac.at/myristate/SUPLpredictor.htm
N-myristoylation	http://web.expasy.org/myristoylator/
S-palmitoylation	http://omictools.com/palmitoylation-site-prediction-category (This site has a summary of available prediction programs)
S-palmitoylation	http://swisspalm.epfl.ch/ (This site has a list of published palmitoylome sequences)