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Current Opinion in Genetics & Development

Lipid-modified morphogens: functions of fats

Josefa Steinhauer and Jessica E Treisman

Despite their location in the aqueous extracellular environment, a number of secreted proteins carry hydrophobic lipid modifications. These modifications include glycosylphosphatidylinositol, cholesterol, and both saturated and unsaturated fatty acids, and they are attached in the secretory pathway by different classes of enzymes. Lipid attachments make crucial contributions to protein function *in vivo* through a diverse array of mechanisms. They can promote protein maturation and secretion, membrane tethering, targeting to specific membrane subdomains, or receptor binding and activation. Additionally, secretion of lipid-modified morphogens of the Wnt and Hh families requires dedicated accessory proteins and may involve their packaging into lipoprotein particles for long-range transport.

Addresses
Kimmel Center for Biology and Medicine of the Skirball Institute and Department of Cell Biology, NYU School of Medicine, 540 First Avenue, New York, NY 10016, United States

Corresponding author: Treisman, Jessica E (Jessica.Treisman@med.nyu.edu)

Current Opinion in Genetics & Development 2009, **19**:308–314

This review comes from a themed issue on
Pattern formation and developmental mechanisms
Edited by Kathryn Anderson and Kenneth Irvine

Available online 11th May 2009

0959-437X/$ – see front matter

DOI 10.1016/j.gde.2009.04.006

Introduction

During development, cells signal to each other using secreted proteins. A class of such proteins known as morphogens can specify distinct cell fates in a concentration-dependent manner, making their graded distribution important for patterning target tissues. Since secreted signaling proteins must travel through the aqueous extracellular environment, it was surprising to discover that several such molecules carry hydrophobic lipid modifications that are added in the secretory pathway. The complex glycosylphosphatidylinositol (GPI) anchor has been shown to tether many secreted proteins with enzymatic, signaling, or adhesive functions to the plasma membrane, restricting their range of action [1]. Members of the Hedgehog (Hh) family of proteins, which act as important patterning signals at many different stages of development, carry a C-terminal cholesterol modification [2•]. In the past few years, fatty acid modifications have been found on the N-terminus of Hh proteins as well as on other secreted proteins that include members of the Wnt family, the Epidermal growth factor receptor (EGFR) ligand Spitz (Spi), and the appetite-regulating hormone Ghrelin [3,4•,5•]. Interestingly, removal of these modifications interferes with the ability of the proteins to carry out their functions *in vivo*. Recent studies have shown that lipid modifications can affect the activity of signaling proteins by altering their secretion, dispersal, or interaction with receptors. However, the consequences of any specific modification are still difficult to predict and must be experimentally determined.

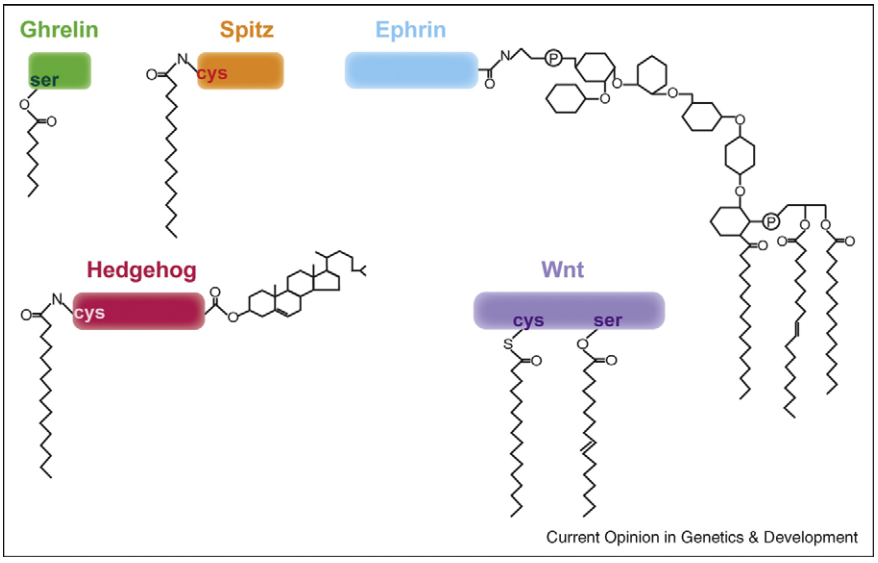
Enzymology of lipid modifications

Lipid modifications can be added to proteins in the secretory pathway by a variety of mechanisms. GPI anchor addition is catalyzed by a 5-subunit transamidase located with its active site in the lumen of the endoplasmic reticulum (ER) [6]. The GPI8 subunit of this enzyme cleaves a hydrophobic signal sequence from the C-terminus of the target protein and transfers a preformed anchor generated through a series of other enzymatic steps [6] (Figure 1). By contrast, cholesterol addition to Hh requires no components other than purified Hh protein itself; the C-terminal intein domain catalyzes proteolytic release of the N-terminal signaling domain and cholesteroylation of its C-terminus [2•].

Recently, the membrane-bound O-acyltransferase (MBOAT) family of polytopic membrane proteins, many of which modify lipid substrates [7–9], has been shown to include members that catalyze fatty acylation of secreted proteins in the lumen of the secretory pathway. The *Drosophila porcupine* (*por*) gene, identified owing to mutant phenotypes very similar to those caused by loss of the Wnt family member *wingless* (*wg*), encodes an MBOAT protein that promotes the hydrophobic modification and secretion of Wg [10–12]. Wnt proteins have two fatty acid modifications, a saturated 16-carbon palmitic acid attached to a conserved cysteine (C77 of Wnt3a) [13••], and a monounsaturated palmitoleic acid attached to a conserved serine (S209 of Wnt3a) [14••] (Figure 1). Por homologs are required for acylation of at least the serine, and possibly both residues [14••,15].

Mutations in a second *Drosophila* MBOAT family member, *rasp* (also known as *sightless*, *skinny hedgehog*, and *central missing*), were identified owing to their defects in Hh and EGFR signaling [16•,17–20]. Both the Hh and Spi ligands

Figure 1



Lipid modifications of secreted proteins. Secreted proteins can be post-translationally modified by the addition of saturated and unsaturated fatty acids, as well as by cholesterol and GPI. The appetite-regulating hormone Ghrelin (green) carries an ester-linked octanoyl group on a serine near its N-terminus. Hh proteins (pink) and the *Drosophila* EGFR ligand Spi (gold) have amide-linked palmitic acids on their N-terminal cysteine residues, and Hh proteins are also modified by cholesterol at their C-termini. Wnts (violet) carry a thioester-linked palmitic acid on an internal cysteine residue as well as an ester-linked palmitoleic acid on a serine residue. GPI linked proteins such as Ephrins (blue) are attached to GPI at their C-termini.

carry essential palmitate modifications on their N-terminal cysteine residues [16•,20,21•,22•] (Figure 1). Unlike palmitate modifications of intracellular proteins, which form thioester bonds with cysteine residues [23], palmitate is attached to Hh by a stable amide linkage to the N-terminal amino group [22•]. The human Rasp homolog Hhat has recently been purified to homogeneity and shown to palmitoylate Sonic hedgehog (Shh) *in vitro*, demonstrating that it is the active acyltransferase rather than a cofactor [24••]. Hhat can modify a peptide corresponding to the first 11 amino acids of Shh, but shows no activity on Wnt or on intracellular palmitoylation substrates [24••]. The finding that Hhatl, an Hhat paralog in which the active site histidine is replaced by a leucine, can act as a competitive inhibitor of Shh palmitoylation by Hhat [25] suggests a potential mechanism for regulation of MBOAT activity *in vivo*.

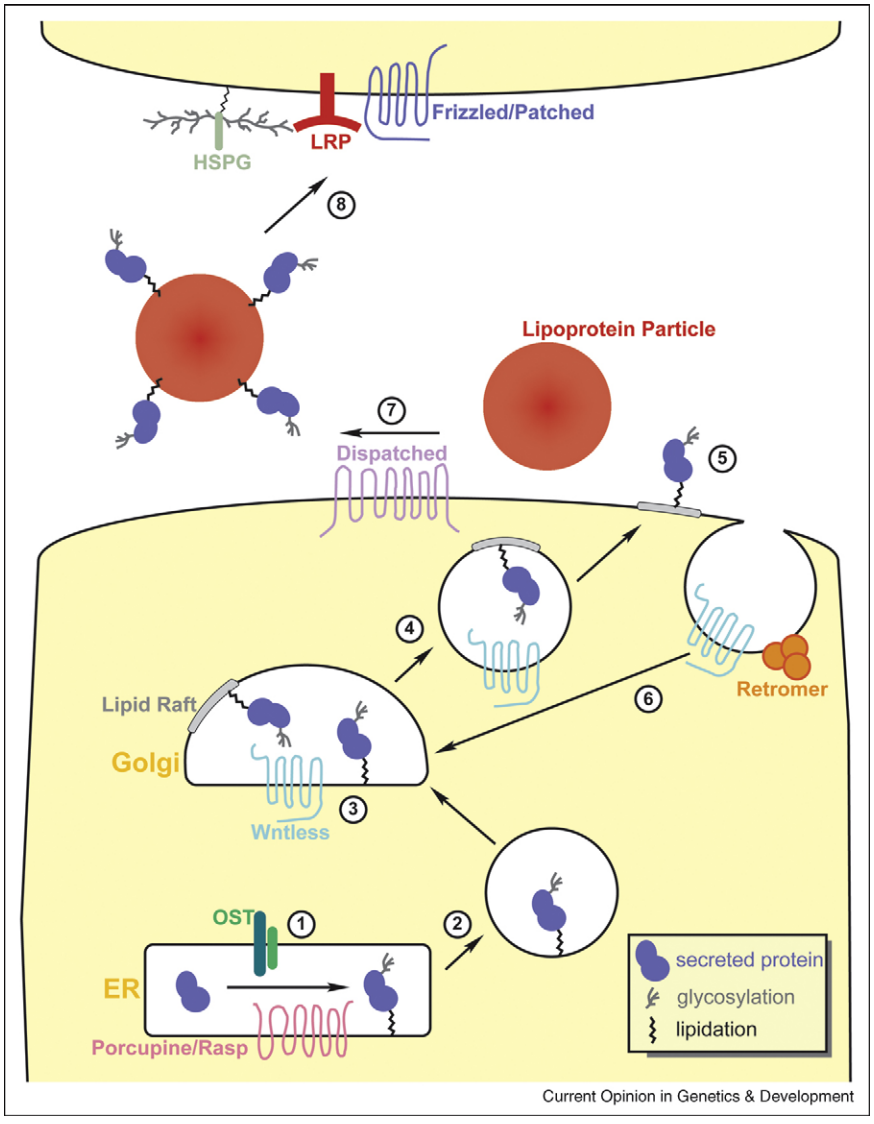
The appetite stimulating and growth hormone-releasing peptide hormone Ghrelin also has an acyl modification essential to its function, octanoylation of serine 3 [26] (Figure 1). The enzyme responsible for this modification is another MBOAT family member, Ghrelin-O-acyltransferase (GOAT) [4•,5•]. The first five amino acids of Ghrelin are sufficient for recognition by GOAT [27], supporting the model that these enzymes recognize fairly short peptide sequences. Further study of the determinants of their substrate specificity may reveal additional extracellular proteins that are candidates for fatty acid modification.

Lipid modifications can tether secreted proteins to membranes

Lipid modification of intracellular proteins promotes their association with membranes [23], suggesting the possibility that lipidation of extracellular proteins might tether them to the outside of the plasma membrane (Figure 2). Indeed, the GPI anchor is believed to play this role, as its phospholipid moiety is stably inserted into the extracellular leaflet of the plasma membrane [1]. There is good evidence that palmitoylation of *Drosophila* Spi also has a tethering function. Wild-type Spi is associated with the surface of cultured cells, and mutation of the palmitoylated cysteine residue results in its release into the medium [16•]. Loss of palmitoylation also increases the range of GFP-tagged Spi movement *in vivo* and results in weaker, but longer range activation of target genes [16•]. Modified Spi, thus, appears to be concentrated on the membrane of producing cells, while unmodified Spi is diluted by diffusion.

Nevertheless, Spi-expressing cells can signal to cells that are not their immediate neighbors, especially in mutants lacking the inhibitor Argos [28]. Some Spi molecules may escape palmitoylation, or this modification may be insufficient for stable membrane tethering. Alternatively, a mechanism for Spi release may exist. GPI-linked proteins can be released after cleavage of the anchor by GPI-phospholipase C, GPI-phospholipase D [29], or other hydrolases such as the Wg antagonist Notum [30], or they can be packaged into membrane vesicles with their

Figure 2



Secretion of lipid-modified proteins. (1) Following translation in the rough ER and translocation into the ER lumen, secreted proteins are glycosylated by the oligosaccharyl transferase (OST) complex. Lipidation can also be catalyzed in this compartment, by MBOAT-family acyltransferases such as Por and GOAT, by GPI transamidase, or, in the case of Hh, by autocatalytic cholesteroylation. Rasp and possibly other acyltransferases may function in later compartments, such as the Golgi. (2) Proper lipidation and glycosylation may be required for ER export. (3) In the Golgi, secreted proteins are sorted for trafficking to the plasma membrane. For many lipidated proteins, this entails partitioning into lipid rafts. Association of Wnts with the cargo receptor Wls also occurs here. (4) Association with lipid rafts may mediate delivery of lipid-modified proteins to the apical plasma membrane. (5) Lipidation stably tethers GPI-linked proteins and Spi to the plasma membrane. (6) Wls is recycled from the plasma membrane to the Golgi for additional rounds of Wnt sorting by the retromer complex. (7) Lipid-modified Wnt and Hh proteins may be packaged into lipoprotein particles for dispersal, possibly with the aid of specialized transporters such as Disp. (8) Lipoprotein particles bearing lipid-modified morphogens may interact with target cells via HSPGs and cell surface receptors such as Wnt receptors of the Frizzled family, the Hh receptor Patched, and/or lipoprotein receptor-related proteins (LRPs).

lipid anchor intact [29]. The palmitate group of Spi is likely to be attached by a stable amide linkage; sequence analysis of Spi secreted by cultured cells suggests that its release may occur by proteolysis rather than depalmitoylation [16•]. Interestingly, cleavage by extracellular metalloproteases was recently implicated in releasing lipid-modified Shh from cell membranes [31].

Lipid modifications may also target proteins to specific membrane subdomains (Figure 2). GPI-anchored proteins such as Ephrins are thought to be concentrated in lipid rafts, regions rich in glycosphingolipids and cholesterol that have been implicated in signal transduction and in endocytosis through the caveolar pathway [32,33]. Lipid rafts form a more ordered phase than the surround-

ing plasma membrane and are resistant to disruption by detergent. Wnt proteins associate with rafts in a Por-dependent manner [11], consistent with the ability of palmitic acid adducts to target intracellular proteins to rafts [23]. However, the double bond in the palmitoleic acid introduces a kink in the acyl chain and would be expected to prevent inclusion in the ordered phase of the raft [23,33]. The opposing effects of the two acyl chains might thus allow for regulation of Wnt localization to rafts. Hh proteins also associate with lipid rafts via both their lipid modifications [21•,34]. This lipidation-dependent localization of Hh to rafts that contain GPI-linked heparan sulfate proteoglycan (HSPG) molecules may enhance its clustering through protein–protein interactions and prepare it for dispersal [35•]. Since lipid rafts are thought to be small, heterogeneous, and highly dynamic [36], different lipid modifications may target proteins to distinct raft populations.

Lipid modifications control secretion

The early observation that *por* mutant cells retain Wg in the ER implicated Por in Wnt secretion [12]. Wnts are cysteine-rich proteins that carry up to four asparagine-linked glycosylations in addition to their lipid modifications. Both types of modifications occur in the ER and might contribute to correct Wnt folding, which is required for its export from the ER [10,37] (Figure 2). Glycosylation appears to be a prerequisite for palmitoylation of mammalian Wnt3a in HEK293 cells [14••,38]. Although the sites of lipid modification are not required for normal glycosylation in these cells [14••,38], *Drosophila* Wg is not glycosylated appropriately in the absence of Por [37], and Por overexpression enhances the glycosylation of multiple Wnts in a variety of cell types [10,37], suggesting that glycosylation and lipidation are intimately linked.

Secretion of mouse Wnt3a requires the serine residue normally modified by palmitoleate [14••], while Wnts lacking the palmitoylated cysteine are released normally from cultured cells. This implicates the unsaturated but not the saturated fatty acid in secretion [13••,38,39]. By contrast, a study of *Drosophila* Wg found that the palmitoylated cysteine is essential for secretion *in vivo* [40]. The discrepancy may reflect more stringent requirements for secretion in polarized epithelial cells than in cells in culture. Furthermore, in *por* mutants, the free sulfhydryl group of the unpalmitoylated cysteine could disrupt the normal pattern of disulfide bonding of cysteine-rich Wnts [41], whereas mutation of the cysteine would not have the same effect. One role for lipid modification thus appears to be to promote Wnt folding and export from the ER.

After ER exit, the association of lipid-modified signaling ligands with lipid rafts may provide a sorting signal for trafficking through the secretory pathway (Figure 2). GPI-linked proteins are segregated into lipid rafts in the Golgi. In some polarized cell types, this directs their trafficking toward the apical side of the cell [42,43]. Regulated raft inclusion of Wnts mediated by the interplay between the two lipid modifications could coordinate their polarized trafficking to the plasma membrane [44]. Indeed, Wg is thought to be secreted from both the apical and basolateral domains of wing disc cells, forming distinct extracellular gradients that may be related to signaling at short range versus long range [44,45]. In addition, release of both Wg and Hh from the plasma membrane is enhanced by the lipid raft scaffolding protein Reggie-1/Flotillin [46].

Most Wnts require the cargo receptor Wntless/Evenness interrupted/Sprinter (Wls) for trafficking from the Golgi to the plasma membrane [47•,48••,49•] (Figure 2). Binding of Wls to Wnt3a requires neither the palmitoylated cysteine nor the glycosylation sites [38], and Wnt3a lacking the palmitoylated cysteine still requires Wls for secretion in cell culture [48••]. Together, these data hint that the requirement for Wls in Wnt trafficking is independent of palmitoylation, although recognition by Wls could involve the serine-linked palmitoleic acid. The retromer complex is also required for Wnt secretion, owing to its function in recycling Wls from the plasma membrane through endosomes to the Golgi [50]. Intriguingly, the recent finding that *Drosophila* WntD, a non-lipidated Wnt involved in dorsal-ventral patterning and innate immunity, does not rely on Wls for secretion suggests that there may be a difference in the exocytosis pathways utilized by lipid-modified and non-lipidated Wnts [51].

Hh secretion is not impaired by mutations in Rasp/Hhat, the Hh acyltransferase, nor by mutations in Hh itself that delete the lipid adducts [17,18,20,21•]. However, cholesterol-modified Hh proteins specifically require the function of the multipass transmembrane protein Dispatched (Disp) for their secretion [52•,53]. Disp is homologous to the RND family of bacterial transporters that transport hydrophobic molecules across membranes, and therefore it is thought to catalyze the release of cholesterol-anchored Hh from the plasma membrane [54] (Figure 2). The specificity of Disp for cholesterol-modified Hh again supports the existence of distinct secretion pathways for lipidated and non-lipidated ligands.

Lipid-modified proteins can be packaged into lipoprotein particles for long-range transport

The question of how membrane-tethered ligands of the Wnt and Hh families can act as long-range morphogens is beginning to be resolved. A clue to the answer came from the discovery that *Drosophila* Wg and Hh copurify and colocalize in imaginal discs with lipophorin, which forms the protein scaffold of lipoprotein particles [55••] (Figure 2). The association with lipoprotein particles, which is likely to be mediated by the lipid modifications on the morphogens, may be important for their transport, since depletion of circulating lipophorin restricts the

range of Wg and Hh signaling in the wing disc [55••]. In mammals, the existence of multiple types of lipoprotein particle adds a further complication; a recent study shows that high density lipoprotein particles (HDLs) but not low density lipoprotein particles (LDLs) can mediate Wnt3a release from cultured mouse fibroblast L-cells [56].

The transport of lipidated proteins by lipoprotein particles is not unprecedented, as GPI-linked proteins such as parasite coat proteins are known to circulate through the body in this manner [29,57], but the regulated use of lipoprotein particles in developmental patterning events may represent a new paradigm. Such a mechanism might explain the findings that the lipoprotein receptor-related proteins Arrow/LRP5/LRP6 and Megalin can act as coreceptors for Wnt and Hh proteins, respectively [58,59], and that the Hh receptor Patched (Ptc) can bind and internalize lipophorin [60]. It remains to be seen whether lipoprotein particles are the only vehicles for morphogen transport; several groups have described the release of lipid-modified Hh and Wg in multimeric aggregates or vesicular structures, some of which might represent alternative transport mechanisms acting in different settings [21•,61,62•,63].

An important question is how signaling proteins are loaded onto lipoprotein particles. Prior work points to the possible involvement of lipid rafts [46] and transmembrane transporters such as Disp [64]. One possibility is that incorporation occurs during the endocytosis and recycling of lipoprotein particles, and that rafts play a role in this process [57]. It is not clear whether more than one morphogen can be incorporated into the same lipoprotein particle; in this case binding of the particle to the receptor for one protein might prevent the second from reaching its target. In support of specificity in lipoprotein particle loading, an artificial GPI-linked form of Hh is not released from the membrane [52•], even though GPI-linked GFP seems to be incorporated into Wg-containing particles [63].

The involvement of lipoprotein particles in Wnt and Hh transport also has implications for the functions of HSPGs in morphogen movement. Cell-surface HSPGs are known to specifically affect the extracellular distribution of lipid-modified Hh and Wg [65,66]. Their effects on the range of morphogen activity may be attributable to their demonstrated affinity for lipoprotein particles [67,68]. HSPGs might also prevent morphogen-loaded lipoprotein particles from entering the circulatory system, which could have dramatic developmental and homeostatic consequences.

Lipid modifications can affect receptor binding or activation

Finally, lipid modifications can enhance the ability of a ligand to bind to or activate its receptor (Figure 2). The palmitoylated cysteine of Wnt molecules is necessary for strong binding to Frizzled receptors [38–40]. Octanoylation of serine 3 of Ghrelin is essential for maximal activation of the growth hormone secretagogue receptor, although its function can be experimentally replaced by other hydrophobic adducts [69]. Interestingly, palmitoylation or other N-terminal hydrophobic modifications of Sonic Hedgehog (Shh) greatly increase its activity in a cell-based assay that does not require transport, without significantly affecting its ability to bind to cells expressing the Ptc receptor [70]. This suggests that unmodified Shh may bind Ptc in a non-productive manner. However, palmitoylation of Spi does not alter its ability to bind to or activate the EGF receptor [16•], indicating that not all lipid modifications contribute to receptor interactions.

Conclusions

Lipid modifications have a surprising variety of effects on extracellular proteins. Lipid modifications of Wnts clearly impact exocytosis, but the specific roles of each post-translational modification with regard to folding, trafficking, raft inclusion, and polarized membrane targeting have yet to be determined. While palmitoylation of Spi appears to tether it to the plasma membrane, in a similar manner to GPI linkage, lipid-modified Hh and Wnt molecules can be transported over a long range *in vivo*. These lipidated ligands require specific cofactors, Wls and Disp, for their trafficking to the cell surface and release from the membrane. The involvement of lipoproteins in Wnt and Hh signaling may explain how lipid-modified signaling ligands are packaged for long-range signaling, dispersed through tissues in a regulated manner, and recognized by receptors on receiving cells, and has the potential to link these processes to the metabolic state of the animal. Future investigations may uncover additional lipid modifications of signaling ligands with novel functional consequences.

Acknowledgements

We thank Sergio Astigarraga, Kerstin Hofmeyer, Kevin Legent, and Jean-Yves Roignant for their critical comments on the manuscript. Work on lipid modifications in the authors' lab has been supported by the National Institutes of Health (grants EY13777 and HD058217 to JET and GM079811 to JS) and by the March of Dimes Birth Defects Foundation (grant #1-FY08-509 to JET). We apologize to colleagues whose work was not cited owing to space constraints.

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