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Lipid Modification of Secreted Signaling Proteins

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Abstract

Proteins of the Hedgehog, Wnt and Epidermal Growth Factor Receptor (EGFR) ligand families are secreted signals that induce concentration-dependent responses in surrounding cells. Although these proteins must diffuse through the aqueous extracellular environment, recent work has shown that hydrophobic lipid modifications are essential for their functions. All three classes of ligands are palmitoylated in the secretory pathway by related enzymes, and Hedgehog also carries a C-terminal cholesterol modification as a result of its autocatalytic cleavage. Palmitoylation is required for Wingless secretion and contributes to the signaling activity of Hedgehog and Wnt3a, but is not required for secretion or receptor activation by the EGFR ligand Spitz. While lipid modifications enhance the long-range activity of Sonic hedgehog, they restrict the range and increase the local concentration of Spitz. We discuss the diverse functions and the possible extent of palmitoylation of secreted ligands.

Keywords

palmitoylation; acyltransferase; lipid; cholesterol; Hedgehog; Wnt; Spitz; Epidermal growth factor receptor; morphogen

Secreted ligands of the Hedgehog (Hh) and Wnt (Wg) families are important regulators of many developmental processes and can act over long distances¹. Hh proteins have been shown to undergo autoproteolytic cleavage accompanied by covalent attachment of a cholesterol molecule to the C-terminal residue of the secreted signaling domain². This finding was followed by studies showing that Hh and Wnt proteins from both *Drosophila* and mouse are palmitoylated by the related acyltransferases Rasp (also known as Sightless, Skinny Hedgehog and Central Missing) and Porcupine (Porc) respectively, members of the membrane-bound O-acyltransferase (MBOAT) family³. Addition of palmitate, a 16-carbon saturated fatty acid, is required for Hh and Wnt function in vivo^{4–10}. Most recently, we reported that Rasp also palmitoylates the Epidermal Growth Factor Receptor (EGFR) ligand Spitz (Spi) and is essential for Spi function¹¹. The palmitoylation site in Hh and Spi is the N-terminal cysteine residue following the signal peptide^{11, 12}; however, Wg is palmitoylated on an internal cysteine residue⁸. Lipid modifications of intracellular proteins often promote their attachment to the plasma membrane¹³, a function not easily reconciled with diffusion to produce a long-range morphogen gradient. In this review, we will compare and contrast the effects of lipid modification on the Hedgehog, Wnt and EGFR ligands (Fig. 1).

Palmitoylation is required for Wg secretion

Palmitoylation appears to be required for the secretion of the *Drosophila* Wnt protein Wingless (Wg). In the absence of either *porc* or the palmitoylation site, Wg protein accumulates in the

cells that synthesize it^{14–16}. Treatment of S2 cells with 2-bromopalmitate, an inhibitor of palmitoylation, also blocks Wg secretion⁹. The effect of palmitoylation on secretion may be due to its ability to target Wg to lipid raft membrane microdomains that form in the Golgi and are preferentially transported to the plasma membrane⁹. Palmitoylation of Ras on the cytoplasmic face of the Golgi likewise promotes its transport to the plasma membrane¹⁷. Alternatively, decreased secretion of unpalmitoylated Wg may result from protein misfolding and retention in the endoplasmic reticulum (ER), where Porc is localized¹⁶. The large number of cysteines in Wg makes misfolding a common response to missense mutations, and lack of palmitoylation would result in a free cysteine¹⁴. Wg N-glycosylation is also dependent on Porc; since Wg is glycosylated post-translationally rather than co-translationally, palmitoylation may be required to anchor it close to the oligosaccharyl transferase complex at the membrane¹⁸.

Other palmitoylated proteins do not require this modification for their secretion. Mutation of the palmitoylation site of mouse Wnt3a does not prevent its secretion but does reduce its activity, suggesting a second function for Wnt palmitoylation⁸. Palmitoylation seems to reduce the secretion of Hh and Spi; both proteins are more efficiently recovered from S2 cell culture medium when unpalmitoylated, with this effect being especially dramatic for Spi^{4, 11}. Consistent with the different effects of palmitoylation on Rasp and Porc substrates, Rasp appears to be localized to the Golgi (Fig. 2) rather than the ER. Unpalmitoylated Spi can reach the Golgi and undergo cleavage there by the protease Rhomboid, suggesting that it is correctly folded^{11, 19}.

The different effects of palmitoylation on Wg, Hh and Spi could also be due to the nature of the palmitate linkage. Wg modification occurs through a thioester linkage that is sensitive to cleavage by hydroxylamine or acyl-protein thioesterase-1^{8, 9}. Thioester bonds are labile, and proteins modified in this way can undergo cycles of palmitoylation and depalmitoylation²⁰. Reversible palmitoylation targets Ras to the cytoplasmic face of the Golgi apparatus, since depalmitoylation releases it from the plasma membrane¹⁷; a similar mechanism might control the localization of Wg within the secretory pathway. In contrast, Hh is palmitoylated on its N-terminal amino acid, and the initial thioester linkage appears to rearrange to form a stable amide bond¹². Spi is also modified on its N-terminal cysteine, and like Hh, shows limited incorporation of radioactive palmitate in cell culture, suggesting that only newly synthesized protein can be labeled¹¹. The irreversible modification of Hh and probably Spi may suggest a function in the extracellular environment.

Palmitoylation is required for receptor activation by Hh and Wnt proteins

Palmitoylation has been shown to enhance the signaling activity of some ligands. In assays in which equal amounts of protein are added to cultured cells, unpalmitoylated Wnt3a, Shh and Hh are less active in promoting pathway activation than the palmitoylated forms^{4, 8, 10, 12}. However, Hh binding to its receptor Patched (Ptc) is not significantly affected by palmitoylation¹². It has not been determined whether palmitoylation affects the binding of Wnt proteins to Frizzled receptors. An effect on Hh signaling activity can also be seen in vivo, as a transmembrane Hh-CD2 fusion protein fails to activate target genes in neighboring cells when expressed in *rasp* mutant wing discs⁴. It is possible that the reduction in activity reflects a loss of access to the membrane domains in which Ptc is located; cholesterol and palmitate modifications have been reported to affect the partitioning of Hh between the apical and basolateral membranes, although the direction of the effect is disputed^{21, 22}. However, a constitutively active chimeric protein in which Hh is directly fused to Ptc²³ also shows reduced activity in the absence of *rasp* (Fig. 3), suggesting that even when bound to Ptc, unpalmitoylated Hh cannot signal productively. Interestingly, other hydrophobic modifications of the N-terminus of Shh can substitute for this effect of palmitoylation²⁴. Finally, mutation of the palmitoylated cysteine to serine confers dominant negative activity on Hh, at least in some

contexts^{22, 25, 26}, suggesting that it may competitively inhibit Ptc binding by wildtype Hh. In contrast, palmitoylation does not affect the ability of Spi to bind and activate the EGFR in vitro, and mutation of its palmitoylation site does not result in dominant negative function in vivo¹¹.

Palmitoylation has divergent effects on transport

Lipid modification can also regulate ligand transport, but it seems to have different effects on different molecules. Paradoxically, hydrophobic modifications appear to increase Hh solubility; loss of either the palmitate or cholesterol modification on mouse Shh reduces its range of action in vivo^{10, 27}. Interestingly, Shh has been found to form a multimeric complex that is distributed over a long range and signals more efficiently than monomeric Shh^{10, 28}. Both lipid modifications are required for Shh incorporation into this complex^{10, 29} and probably also for Hh incorporation into a similar complex^{10, 21}. Recent results in *Drosophila* suggest the possibility that this complex is a lipoprotein particle. Apolipoproteins copurify with Hh as well as with Wg, and are required for long-range activity of Hh, although their effect on Wg activity appears weak³⁰. Incorporation of Hh into such a complex may be mediated by Dispatched, a transmembrane protein required for the secretion of cholesterol-modified Hh^{31, 32}. Transport of the complex in vivo requires heparan sulphate proteoglycans (HSPG)³³, which may be linked to Hh by Shifted, a secreted Wnt Inhibitory Factor homologue^{34, 35}.

Palmitoylation appears to have the opposite effect on the transport of Spi. Spi normally acts as a short-range ligand, for example in the pairwise recruitment of photoreceptors to an ommatidium³⁶. Misexpression of a truncated form of Spi that does not require cleavage by Rhomboid usually activates target gene expression only in immediately adjacent cells^{11, 37}. However, misexpressed unpalmitoylated Spi can activate weak target gene expression over a long range¹¹. Visualization of tagged Spi molecules suggests that palmitoylation tethers Spi to the plasma membrane of producing cells, preventing its release into the extracellular space¹¹. This would result in a high local concentration of Spi, allowing it to reach the threshold necessary for target gene activation. In contrast, unpalmitoylated Spi would diffuse away and become diluted out, forming a shallower gradient sufficient for target gene activation only under conditions in which Spi is overexpressed¹¹. It is unclear whether Spi can directly bind its receptor while anchored to the membrane by palmitoylation, since Spi is synthesized as a transmembrane protein precursor that is inactive until it is cleaved by the protease Rhomboid¹⁹. Intriguingly, Spi isolated from S2 cell culture medium has been shown to initiate at methionine 45¹¹, suggesting that local proteolytic cleavage might generate a soluble, active form of Spi.

Can these apparently different effects of lipid modification be reconciled? One possibility is suggested by a recent paper that uses mathematical modeling to simulate Shh signaling in the neural tube³⁸. The authors obtain the counter-intuitive result that reducing the diffusivity of Shh, an effect that could be produced by lipid modification and/or HSPG binding, extends its effective range of signaling³⁸. The basis for this effect is that untethered Shh diffuses away rapidly and becomes diluted out, failing to reach the threshold required for target gene expression at a distance from the source. Lipid modifications have been shown to reduce the secretion of Hh from cultured cells, supporting the idea that they promote membrane tethering^{22, 39}. This model could explain why loss of cholesterol or palmitate modification of Shh specifically prevents long-range signaling in the mouse limb bud^{10, 27}. Initial reports suggested that removal of cholesterol from *Drosophila* Hh increased its range of action in the wing disc³². However, this may have been due to its expression in the squamous peripodial cell layer overlying the disc proper, as a reduced range has been seen when Hh lacking cholesterol is restricted to columnar disc epithelial cells²². Interestingly, the range of distribution of GFP-tagged Hh proteins in the wing disc and the range at which they activate

low-threshold target genes is increased when they lack the cholesterol or palmitate modifications, although the range at which they can activate high-threshold target genes is decreased^{21, 22, 40}. However, the loss of palmitate causes a much greater functional impairment than loss of cholesterol, suggesting an additional effect on Hh activity as discussed above. It remains to be determined whether incorporation into lipoprotein particles indeed enhances Hh transport, or increases the functional range of Hh by restricting its diffusion.

Lipid modification is only one among a number of mechanisms that have evolved to restrict the range of diffusible ligands. Hh is sequestered and endocytosed by its receptor Ptc; induction of high-level *ptc* expression by Hh signaling provides a negative feedback mechanism to limit Hh diffusion^{41, 42}. The Wg receptor Dfrizzled2 seems to have the opposite effect, stabilizing Wg to expand its range of function⁴³; however, a receptor-independent endocytic pathway restricts Wg spreading⁴⁴. Endocytosis and recycling by producing cells also contributes to high Wg levels near the source⁴⁵. Some ligands are sequestered by secreted proteins rather than receptors; for example, Spi signaling induces expression of the feedback inhibitor Argos, which binds to Spi and prevents it from binding its receptor^{46, 47}. In vertebrates, Shh induces the antagonistic Hedgehog-interacting protein (Hip)⁴⁸ and a variety of secreted antagonists modulate Wnt function⁴⁹. In contrast to these mechanisms, restricting the range of a ligand by lipid modification increases the local concentration of the active form, enhancing signaling. Loss of Argos is insufficient to compensate for the loss of palmitoylation of Spi¹¹ and reducing Ptc dosage does not rescue loss of Shh palmitoylation¹⁰, suggesting that palmitoylation is necessary to prevent ligand dilution even in the absence of feedback inhibitors.

Are other ligands palmitoylated?

The discovery that Hh, Wnt and Spi proteins are palmitoylated was recent and unexpected, and this modification appears to have a diversity of functions. It is thus tempting to speculate that other secreted ligands may be palmitoylated as well. The basis for acyltransferase specificity is not clear, as the two known Rasp substrates, Hh and Spi, share little sequence homology beyond some basic residues in the vicinity of the palmitoylated cysteine. It is therefore possible that Rasp and Porc have additional substrates. Based on phenotypic analysis, Rasp appears likely to modify the related EGFR ligands Gurken and Keren, but not the long-range Neuregulin-related ligand Vein¹¹. *rasp* maternally and zygotically mutant embryos show normal mesoderm spreading and tracheal branching, suggesting that *rasp* is not required for the function of the Fibroblast Growth Factor (FGF) homologues Pyramus, Thisbe and Branchless^{50, 51} (J. Steinhauer and J.E.T., unpublished data). We have also found that *rasp* is not essential for the function of the BMP family ligand Decapentaplegic (Dpp), as Dpp is able to activate its target genes when misexpressed in *rasp* mutant wing discs (Fig. 4). However, other ligands remain to be tested. Rasp and Porcupine belong to the MBOAT family of proteins, which also includes enzymes that acylate lipid substrates³. Several uncharacterized acyltransferases of the MBOAT family are present in the *Drosophila* and vertebrate genomes. Future investigations will reveal whether they also modify ligands important for development.

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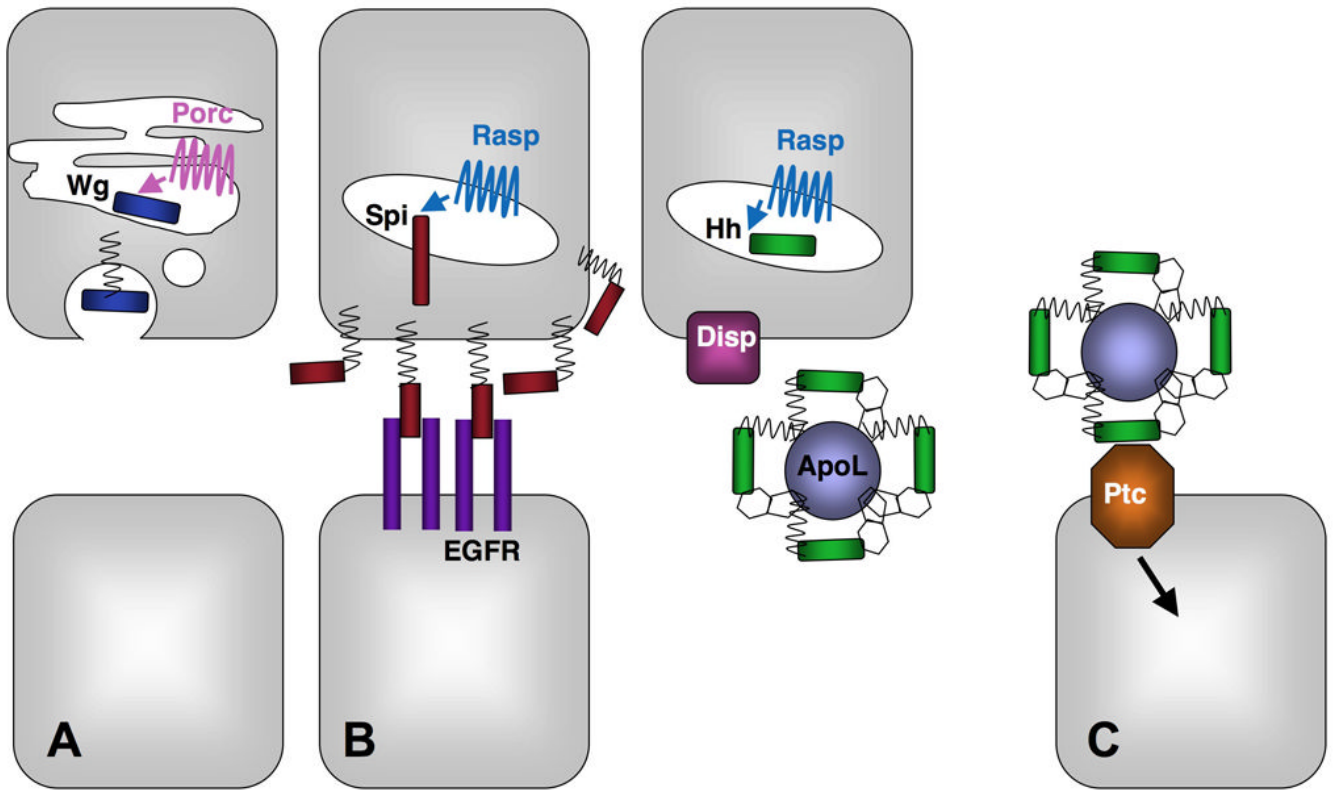


Figure 1. Different effects of lipid modifications on Spi, Hh and Wg

Ligand-producing cells are shown in the upper row and ligand-receiving cells in the lower row. Porc in the ER palmitoylates Wg on an internal cysteine (A), while Rasp in the Golgi palmitoylates Spi and Hh on their N-terminal residues (B, C). Palmitoylation of Wg enhances its glycosylation, lipid raft targeting and secretion (A). Palmitoylation of Spi increases its association with the plasma membrane of producing cells and raises its local concentration to the threshold necessary to activate the EGFR (B). Palmitate and cholesterol modifications on Hh promote its incorporation into multimers that may contain Apolipoprotein (ApoL) and enhance its long-range activity. Palmitoylation is also required for Hh to signal productively when bound to Ptc (C).

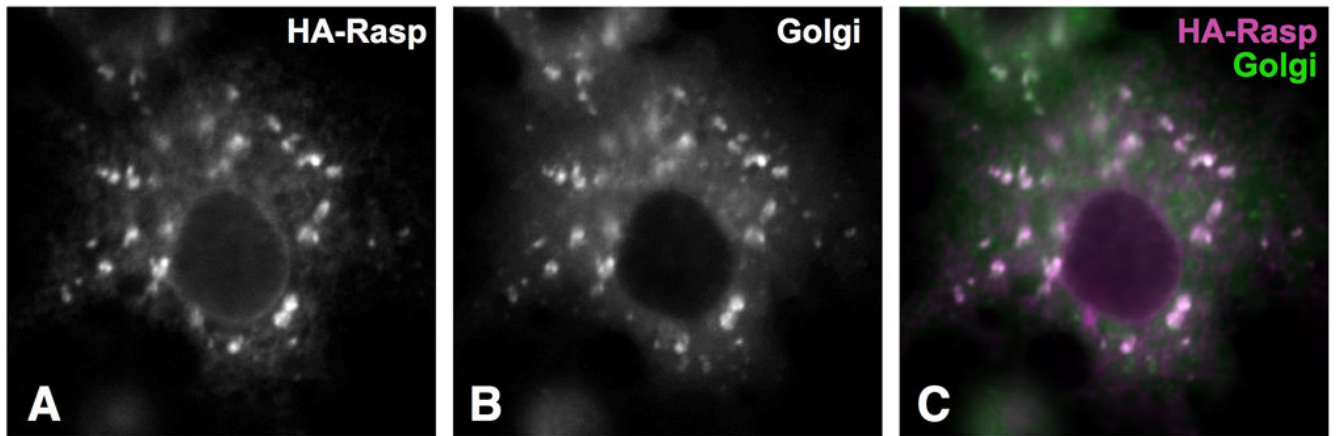


Figure 2. Rasp is present in the Golgi in *Drosophila* S2R+ cells
(A–C) show a cell transfected with *actin-GAL4*, *UAS-rasp-HA*¹¹ and the Golgi marker *UAS-dGRASP65-GFP* (kindly provided by Henry Chang). Anti-HA staining (A, magenta in C) colocalizes with GFP (B, green in C).

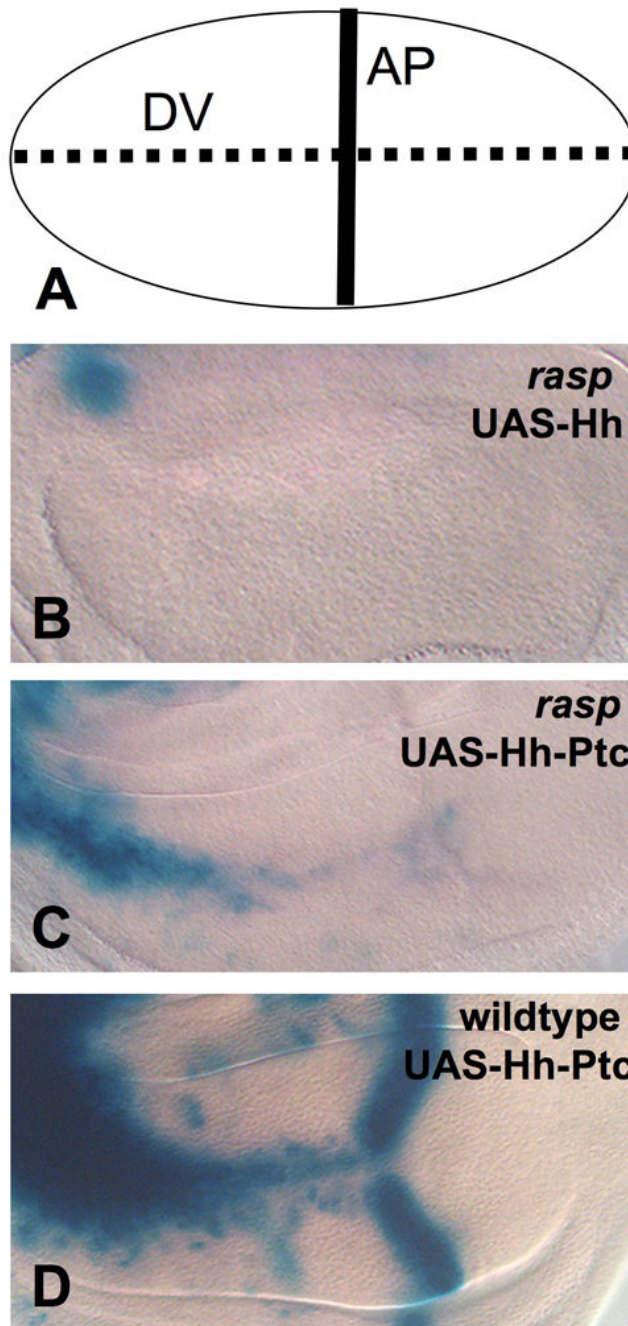


Figure 3. A Hh-Ptc fusion protein shows reduced activity in *raspl* mutant wing discs
 (A) shows a schematic of the wing pouch. The solid line indicates the anterior-posterior (AP) compartment boundary where endogenous *dpp* is expressed, and the dotted line indicates *vestigial* (*vg*)-GAL4 expression at the dorsal-ventral (DV) boundary. (B–D) show third instar wing discs carrying *vg*-GAL4 and *dpp-lacZ* and stained with X-Gal. (B–C) *raspl*^{T392}/*raspl*^{T802}; (D) wildtype. (B) expresses UAS-*hh* and (C–D) express UAS-*hh-ptc*²³. All discs were stained in parallel for the same length of time. The Hh-Ptc fusion activates *dpp* expression to a slightly greater extent than Hh in the absence of *raspl*, but its activity is lower than in wildtype discs. Note that endogenous *dpp* expression at the AP boundary is lost in *raspl* mutant

discs due to the lack of palmitoylation of endogenous Hh⁴⁻⁷. The expression pattern of *vg-GAL4* is not altered in *rasp* mutant wing discs (¹¹ and see Fig. 4).

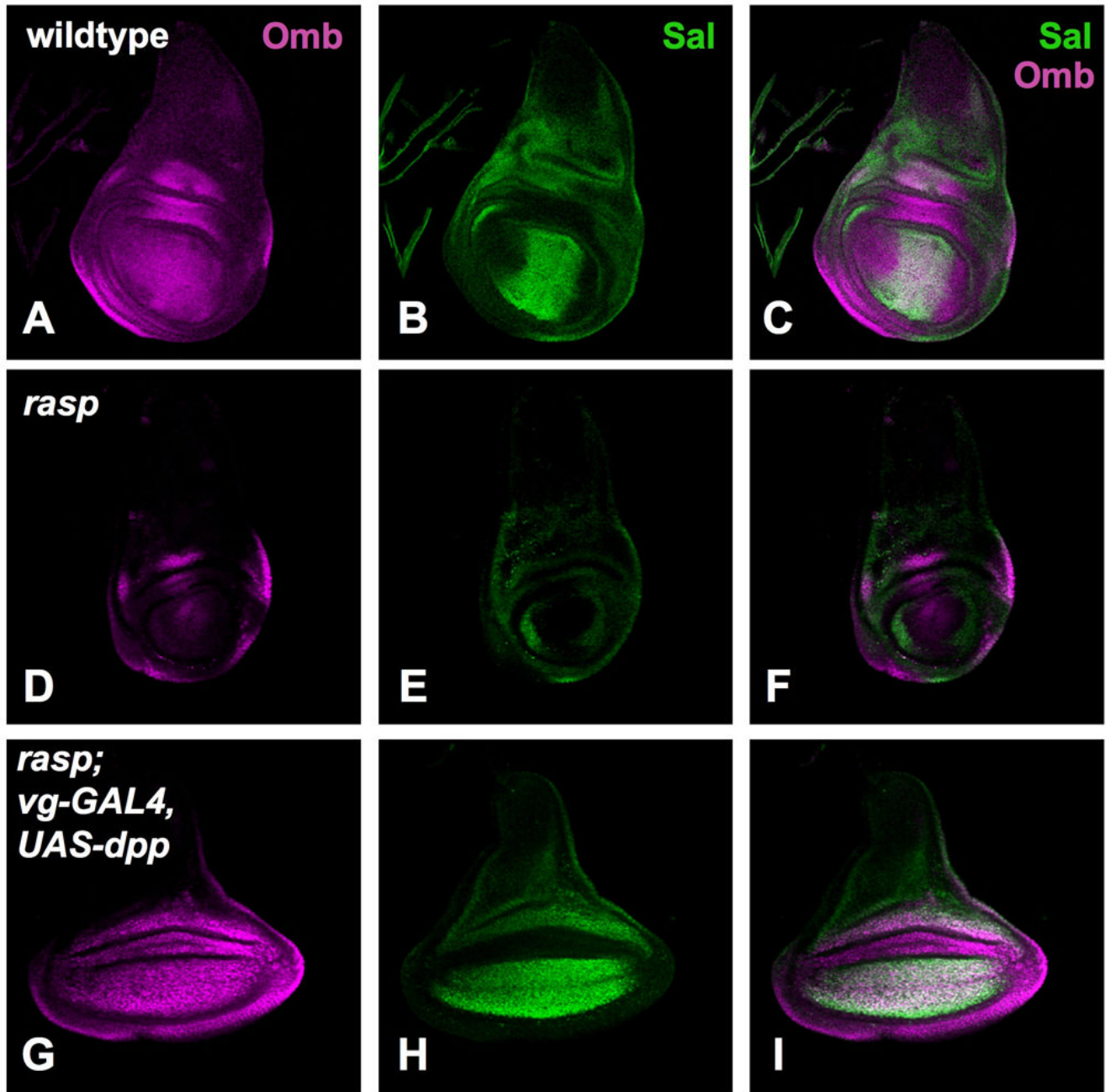


Figure 4. Dpp is active in the absence of *rasp*

All panels show third instar wing discs. (A–C) wildtype; (D–F) *rasp*^{T392}/*rasp*^{T802}; (G–I) *rasp*^{T392}/*rasp*^{T802}; *vg-GAL4/UAS-dpp*. The discs were stained to show expression of the Dpp target genes *optomotor-blind* (Omb; magenta in A, C, D, F, G, I) and *spalt* (Sal; green in B, C, E, F, H, I). *dpp* is not expressed in *rasp* mutant discs due to defective Hh signaling (⁶ and see Fig. 3); however, when misexpressed at the dorsal-ventral boundary of the wing disc it is able to activate *sal* and *omb* expression. Transfections and X-gal and antibody stainings were carried out as described¹¹.