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Wnt and Hedgehog: Secretion of Lipid-Modified Morphogens

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Abstract

Morphogens are signaling molecules produced by a localized source, specifying cell fate in a graded manner. The source secretes morphogens into the extracellular milieu to activate various target genes in an autocrine or paracrine manner. Here we describe various secreted forms of two canonical morphogens, the lipid-anchored Hedgehog (Hh) and Wnts, indicating the involvement of multiple carriers in the transport of these morphogens. These different extracellular secreted forms are likely to have distinct functions. Here we evaluate newly identified mechanisms that morphogens use to traverse the required distance to activate discrete paracrine signaling.

Multiple Carriers for Hh and Wingless (Wg) Transport

Morphogens play a major role in tissue patterning by activating various target genes in a spatiotemporally regulated manner [1]. They are predominantly produced by a localized source and function in a concentration-dependent manner. Interpretation of these concentration gradients has received increased attention [2]. Morphogen-mediated cell-fate determination requires coordination between several cellular processes such as morphogen production and secretion by the source, transport across several cell diameters, efficient receptor binding, and processing by signal-receiving cells [3,4].

Lipid-modified Hh and Wg are membrane-anchored morphogens that play major roles in tissue patterning and maintenance and in disease progression. First identified in *Drosophila*, these morphogens are conserved in metazoans [5,6]. In *Drosophila* they activate short- and long-range paracrine signaling to pattern the larval imaginal discs (e.g., wing discs; Figure 1A,B), which ultimately give rise to the adult body parts [7,8]. In vertebrates Hh [i.e., Sonic hedgehog (Shh)] and the Wg homologs (Wnts) act at short and long range to pattern the developing neural tube [9] (Figure 1C), limbs [10], craniofacial features [11,12], and endocrine system [13,14]. In adult systems these morphogens play a major role in stem cell maintenance [15], tissue repair [16], neural connection rewiring [17], and metabolism [18]. They are also hyperactivated in several carcinomas [19].

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Hh-family proteins undergo a series of post-translational modifications in the secretory pathway *en route* to the plasma membrane (PM), to generate a cholesterol- and palmitate-modified signaling protein [20–22]. Similarly, Wg and most Wnts are modified by palmitate or palmitoleic acid [23–25]. Despite being lipidated and membrane tethered, these proteins activate signals several cell diameters away from their source (Figure 1). This has raised two questions. (i) How is a lipid-anchored molecule released from the PM? (ii) How are the hydrophobic lipid tails of this molecule shielded in the aqueous milieu, enabling its transport in the extracellular space? Over the years several groups have made attempts to address these issues.

Extracellular Hh- and Wg-family proteins have been identified in association with various carriers, such as exovesicles or exosomes [26–32], lipoproteins [33,34], and cytonemes [31,35–39]. Hh proteins are also released by matrix metalloproteases (MMPs) in a lipid-free form [40]; such a mechanism for secretion of Wg proteins has not yet been reported except in the context of WntD, a naturally non-lipid-modified homolog [41]. Extracellular Hh and Wg are classified into three main forms: exovesicles (exosomes or microvesicles) and lipoprotein-associated and soluble forms. Several molecular players, such as Dispatched [42], Scube2 [43], and flotillin-2 [44] for Hh and Evenness interrupted (Evi)/Wntless [27,29], Swim [45], and Afamin [46] for Wg secretion, have also been identified as involved in the efficient release of these morphogens. Once they have been secreted into the extracellular milieu, cell-surface heparan sulfate proteoglycans (HSPGs) are known to facilitate their transport to neighboring signaling-competent cells [47]. However, a composite understanding of how these players function with respect to each other or with respect to the various secreted forms is missing. Here we discuss whether the different modes of release of membrane-anchored morphogens generate distinct extracellular forms with different signaling capacities.

Exosomes as Morphogen Carriers

Exosomes are secreted vesicles of 30–100 nm in size. Exosome-based intercellular interactions have been implicated in immunological processes, neuron–glia and tumor–stroma crosstalk [48], and morphogen-based paracrine signaling [26–32]. Exosomes are usually generated by the sorting of endocytic cargo into multivesicular bodies (MVBs). These proceed via an endosomal sorting complex required for transport (ESCRT)-dependent or -independent mechanism [49] (Box 1).

Recently, exosomes have been identified as carriers of Hh and Wg [26–32]. While it has been proposed that Wg secretion in *Drosophila* cells does not require the canonical players involved in MVB formation [i.e., ESCRT, Alix, or ceramide function [29] (unpublished data in [50])], Wnt-3 secretion by HEK cells [28] requires ESCRT function. Regardless, these molecules are first sorted into intraluminal vesicles (ILVs) in MVBs for their exosomal secretion. Since most of the cargo that accumulates in the MVB is destined for degradation [51] (Box 1), this raises the question of how the cells distinguish between the secretory and the degradative fate of these ILVs/MVBs. Given that exosomes ferry morphogens and play a significant role in disease progression via molecules on and in them [52], it will be valuable to identify these mechanisms and regulators to better understand morphogen secretion.

Exovesicles as Hh Carriers

The first evidence for secretion of Hh on exosomes came from work done in *Caenorhabditis elegans*. Here it was demonstrated that Hh-related peptides required for larval cuticle patterning are detectable on exosomes and require V0-ATPase activity [53]. A significant body of work has subsequently highlighted that cell lines and tissues secrete functional Hh molecules on exosomes [26,30–32]. Exovesicular release of Shh has also been reported in mouse embryos [54]. In wing discs, ESCRT proteins such as Tsg101 (ESCRT-I), Vps22 (ESCRT-II), Vps32 (ESCRT-III), and Vps4 are involved in Hh release and target gene activation [26,31]. Further, Hh proteins are sorted into at least two types of exosomal pools using differential ultracentrifugation from a *Drosophila* cell line, a human cell line, and primary chick notochord cells in culture. These biochemically distinct pools have different sedimentation capabilities and have distinct diffusion properties as monitored by fluorescence correlation spectroscopy as well as different compositions (proteins and RNAs) [30,32]. While the classical exosomal pool contains proteins capable of sorting in cholesterol–sphingolipid-rich microdomains, such as flotillins, annexins, and integrins; the newly identified pool of exovesicles appears to be devoid of these proteins [30,32]. Therefore, these two different types of exovesicle might be a result of microdomain segregation on the MVB-limiting membrane [30]. Notably, these vesicles activate different differentiation programs during ventral neuronal progenitor specification [30]. Here, only the classical exosomal pool was able to activate Shh-target genes (ventral neuronal progenitors), while the newly identified pool of exovesicular Shh failed to activate Shh-target genes. However, the mechanism and molecular players involved in generating these distinct pools have not been identified and hence their ability to travel or signal across developing tissues has not been resolved. To summarize, Hh is secreted on different types of exovesicles across different cell types and this difference has functional significance.

Microvesicles or Exosomes?

Besides exosomes, Hh molecules may be released on microvesicles that are generated by budding directly from the PM (Figure 2 and Box 1). Distinguishing PM budding from MVB-mediated exosomal release of Hh molecules is not straightforward since the core ESCRT machinery (ESCRT-I, -II, and -III) and Vps4 [55,56] are also implicated in microvesicle formation [57]. However, regulators of endocytosis such as Rab proteins are not involved in microvesicle biogenesis but regulate exosomal release of cargo by facilitating their sorting into MVBs.

Although several lines of evidence suggest that Hh exovesicles are derived from endocytic processing, an alternative possibility of secretion via microvesicles has also been proposed [38]. Evidence for the role of exosomes may be summarized as follows. (i) In S2 cells, Hh molecules are endocytosed into Rab7⁺Lamp⁺ endosomes by producing cells [32] and sorted onto ILVs (immune-electron microscope (EM)-based analysis; [31,32]) suggesting that they traffic to specific MVBs. This endocytosed Hh appears to be actively sorted away from degradation as it does not traffic towards tubular lysosomes [32], where degradation of endocytosed cargo occurs [58], nor is its trafficking affected by loss of function of a key lysosomal regulator, Deep orange, in wing discs [42,59]. Sorting of Hh-GFP as well as of endogenous Hh into the MVBs of Hh-producing cells has also been demonstrated in wing

discs [31,42]. (ii) In cell lines, perturbing endocytosis by downregulation of Rab5, a regulator of early endocytosis, as well as Shibire, a *Drosophila* dynamin, and of Rab27, a regulator of MVB fusion with the PM, quantitatively affects the secretion of Hh on exovesicles [30,32]. Similarly, in wing discs perturbation of Rab5 function in Hh-producing cells results in intracellular accumulation of endogenous Hh, and reduced Hh signaling range [42,59]. Furthermore, a mutant of Hh (HhK132D) that is defective in oligomerization and long-range signaling [60] was defective in endocytosis and exovesicular secretion [32]. Last, overexpression of Dispatched (Disp), a regulator of Hh secretion, caused it to localize along with Hh on endosomes in the posterior domain of wing discs [42] and mediate endocytosis of Hh [59].

An alternative model suggests that Disp-dependent endocytosis of Hh followed by Rab4-dependent recycling is necessary for a secondary secretion event [59], which could be Hh release on microvesicles via PM budding. This idea is supported by the surface accumulation of Hh on the expression of a dominant-negative form of the key player involved in scission of the vesicles in wing discs, Vps4 [26] (Figure 2). In separate studies RNAi-mediated down-regulation of Vps4 as well as an ESCRT-III (Shrub) not only affected exovesicular secretion of Hh but also resulted in its intracellular accumulation in Hrs⁺ and Rab7⁺ endosomes in wing discs [32]. This suggests a MVB-dependent route for exosomal secretion of Hh. This observed discrepancy may be attributed to a difference in how the ESCRT machinery was perturbed (compare [26] and [32] for details). Experiments to track cell-surface Hh colocalization with various players, such as endogenously tagged Rab proteins [61], will be necessary to further dissect and understand the cellular events and molecular players involved in Hh secretion.

The need for such a complicated process for Hh release following endocytosis remains to be uncovered. A potential clue comes from studies with a Hh isoform that is incapable of forming higher-order oligomers (HhK132D) [60]. This isoform is also incapable of being efficiently packaged into exovesicles due to its poor endocytic capacity [32]. It is conceivable that endocytosis may be necessary to ensure that Hh molecules are organized and packaged with correct binding partners to generate exovesicles with efficient signaling and cell-fate determination capabilities [30,52].

Wnts and Exovesicles

Wg was the first morphogen found to be associated with and transported on extracellular vesicles that are similar to exosomes, termed argosomes [62]. However, later it was suggested that argosomes could be circulating lipoprotein particles (Lpps) on which Wg, Hh, and GPI-anchored proteins can be loaded [34]. One of the first pieces of evidence of exosomal release of Wg was obtained from studies of the *Drosophila* neuromuscular junction (NMJ). Evi, an eight-pass transmembrane protein necessary for Wg secretion, was found to be important for trans-synaptic transfer of Wg at the NMJ [63]. Evi was secreted on exosome-like vesicles at the NMJ and from cultured S2 cells that required Rab11 and syntaxin-1A [50]. Subsequently, cell-based assays showed the presence of Wg on exosome-like vesicles, secretion of which also required Rab11 function [28,29]. In wing discs, however, RNAi-mediated depletion of Rab11 failed to perturb the Wg gradient or target

gene activation [29]. These results suggested that, unlike at the NMJ and in cell lines, Rab11 does not regulate the secretion of Wg on Evi exosomes in wing discs [29].

Evi and Wg independently colocalize with CD63-GFP (a marker of exosomes) in wing discs and cofractionate in biochemical assays; however, colocalization between Evi and Wg is extremely low, suggesting that they are not secreted on the same exosomes [28,29]. It is likely that Wg exosomes produced in wing discs and at the NMJ are distinct. Alternatively, Evi might be involved in the trafficking of Wg from the Golgi to the PM [64]. In both fly and mammalian cell lines, Wg localizes in internal compartments containing ESCRT-0 and -I [28,29] and knockdown of ESCRT-0 components [hepatocyte growth factor-regulated tyrosine kinase substrate (HGS)] affects exosomal Wg secretion [28]. Secretion of Wg exosomes proceeds via a MVB-dependent pathway since blocking of endosomal acidification (using NH₄Cl or bafilomycin A1) and preventing its maturation affects the proper sorting of Wg into ILVs and blocks its secretion in both cell lines and wing discs [28]. In addition, the soluble NSF attachment protein receptor (SNARE) protein Ykt6 affected secretion of Wg, CD63, and CD81 – known exosomal markers in wing discs [28]. Wg-producing cells expressing Ykt6 RNAi in wing discs also resulted in intracellular trapping of Wg, reduction of its extracellular levels, and alterations in its spreading [28]. Thus, it appears that different molecular regulators govern exosomal release of Wg at the larval NMJ, in cell lines, and in wing discs (Figure 2).

Soluble Forms of Hh

The release of soluble forms of vertebrate Hh molecules via disintegrin and MMP domain protein (ADAM)-17 activity suggests that ADAM-17 is involved in the generation of a lipid-free, soluble, and biologically active pool of Hh proteins from cell lines [40]. ADAM-17 not only removes cholesterol and palmitate modifications but also cleaves the CW domain from the N terminus of Hh [65]. While the MMP-cleaved lipid-free Shh is signaling competent in a cell-based assay – namely, C3H10T1/2 osteoblast precursor cell differentiation and chick chondrocyte differentiation assay – it is unproven whether this form of Shh also exists under physiologically relevant conditions and contributes to tissue patterning and maintenance. However, it is tempting to speculate that this form of Hh might be unable to travel long distances in a directed fashion since it lacks the CW domain required for interaction with HSPGs and long-range transport. Soluble Hh molecules are also found *in vivo* in *Drosophila* hemolymph and in wing discs [66]. However, this is a non-sterol-modified but palmitoylated, lipoprotein-free form called HhN* (sterol-free form). Based on relative differences in the electrophoretic mobility of these forms, it has been speculated that HhN* might be distinct from the MMP-cleaved lipid-free-form [66]. Secretion of HhN* in the hemolymph was facilitated by expression of Hh in the fat body and simultaneous suppression of lipoprotein production. HhN* is monomeric/dimeric and signaling competent [66]. A soluble form of Hh is also detected when Hh is expressed in cell lines grown in the presence of serum factors [32]. How HhN* is generated remains unknown and its mechanism would clarify how many types of signaling-competent, soluble forms of Hh exist.

Hh proteins have also been secreted as multimeric complexes from *Drosophila* salivary glands, a *Drosophila* cell line (C18 cells), and vertebrate cell lines (CHO, STO, HeLa, HEK293T) [67–70]. Genetic evidence suggests that multimeric forms of Hh could represent complexes bearing different ratios of HSPG and Hh molecules [71]. However, the nature and origin of these complexes remain elusive.

Lipid-less forms of Wg/Wnt proteins have not been identified yet; however, proteins such as Swim, a Lipocalin family of extracellular transport proteins, and Afamin, a serum glycoprotein (α -albumin), are known to bind lipidated Wnt and generate soluble and active forms of Wg/Wnt proteins [45,46].

Lipoproteins as Morphogen Carriers

Hh and Wg are found on Lpps. The presence of Hh on Lpps and their role in ferrying Hh molecules in *Drosophila* wing discs has been explored [34,66]. Unlike the role of Disp, Lpp-mediated Hh release from producing cells does not require its cholesterol anchor; GPI-anchored GFP as well as Wg is transported on Lpps to a similar extent [34]. Using biochemical characterization and immunostaining studies of *Drosophila* hemolymph as well as mammalian-cell-based assays, it has been confirmed that a subset of secreted Hh and Shh is associated with lipoproteins.

When Hh is overexpressed in fat bodies (tissue that generates Lpps), it is efficiently released on Lpps but is only partly functional. Overexpression of Hh resulted in expansion of the Hh-protein gradient and stabilization of the full-length cubitus interruptus (Ci) across wing discs but failed to trigger target gene activation [66]. Lpp Hh competes with endogenous Hh produced locally by the wing discs, resulting in repression of transcriptional targets of Hh signaling (i.e., Col [66]). These results suggest that Lpp Hh in circulation is incapable of eliciting a complete Hh signaling response. However, under physiological conditions Lpp Hh, present in hemolymph, is mainly derived from enterocytes present in the midgut [72]. Hh secretion in the hemolymph is dependent on the nutritional status of the cells and is upregulated on starvation. Larval growth rate and timing of pupariation are controlled by the levels of circulating Hh [72]. Circulating Hh signals to the fat body cells to control larval development and to the cells of the prothoracic gland to regulate pupariation. Here, high levels of Hh inhibit the production of ecdysone and makisterone A, delaying pupariation. While this study ascribes a novel function to Lpp Hh, the precise regulation of circulating Hh and its influence on developmental stages remains unclear. Under physiological conditions Lpp Hh might play a hormonal role, and it does not seem to be involved in target gene activation for tissue patterning. Thus, Lpp Hh seems to be involved in noncanonical signaling, while sterol-free Hh (i.e., HhN*) can activate canonical signaling. Taken together these studies indicate that the different forms of Hh influence its signaling abilities.

Morphogens Associated with Cytosomes

In 1999 cytosome-based morphogen transport was proposed for the first time [73]. Cytosomes are long, filopodium-like structures that assist in the transport of not only membrane-anchored Notch, Wg, and Hh [31,35,39] but also secreted morphogens such as

Decapentaplegic (Dpp), fibroblast growth factor (FGF), and epidermal growth factor (EGF) [74].

It has been demonstrated that endogenous Hh or Hh-GFP puncta organize on long, thread-like projections perpendicular to the AP boundary, resembling cytonemes [35]. Other overexpressed Hh-pathway components, such as Interference Hh (Ihog), Brother of interference hedgehog, Disp, and Dally-like protein (Dlp), also localize on these structures [35,75]. Cytonemes fail to cross mutant clones deficient in HSPG (cell-surface or extracellular matrix proteoglycans) production [35], suggesting that interaction between HSPGs and cytonemes is necessary for cytonemal extension. Interestingly, HSPGs are also necessary for long-range transport of Hh. HSPGs form visible clusters at the cell surface [60] and since Dlp decorates cytonemes, it is possible that homotypic interactions between HSPGs on the cytonemes and those in the extracellular matrix could determine the stability, extension, and localization of cytonemes.

Cytoneme-based transport of Hh molecules is likely to be conserved and has also been implicated in Shh transport in chick embryos [36]. Thus, cytonemes and HSPGs might work together to transport morphogens across cell layers. CD63-GFP (an exosomal marker) [31] and Ihog-CFP (Hh coreceptor) [35] were visualized on cytonemes as punctate structures; Ihog-CFP in wing discs was also identified on cytoplasmic protrusions and on exovesicles in the extracellular space in an ultrastructural study [35]. Thus, exosomes might use cytonemes as tracks to travel across tissue. Cytonemes have also been demonstrated in the transport of Wg/Wnt molecules across developing tissues [39,76]. In zebrafish live embryos as well as in fibroblast cells, overexpressed Wnt-8a-GFP was observed on cytonemes. Variations in filopodial length and frequency could influence the distribution of Wnt-8a and neural plate patterning [76]. In *Drosophila*, flight muscle progenitors (myoblasts) extend cytonemes containing Frizzled (Wg receptor) directly to Wg-producing cells in the wing disc [39]. This cytoneme-mediated Wg signaling in myoblasts can indirectly influence air sac primordium patterning [39].

Most studies pertaining to cytoneme visualization and correlation with the presence of morphogens and their receptors or coreceptors on these membrane extensions are performed under overexpression conditions. Hence, it remains to be seen whether this is the preferred mode of morphogen transport under physiological conditions.

Distinct Functions for Different Carriers?

The lipid-anchored nature of some forms of Hh and Wg raised the question of how these proteins signal over long distances and what carriers they may use. Hh and Wg signaling employ similar carriers for their transport across cell boundaries, such as Lpps, flotillin-based carriers, cytonemes, and exosomes. Are specific extracellular forms involved in short-versus long-range delivery of morphogens? There appears to be no correlation between signaling range and type of carrier in the available literature. For example, it has been observed that downregulation of Ykt6 (V-SNARE) affects exosomal secretion of Wg and reduces expression of Senseless (Sens), a short-range signaling target, suggesting that exosomes are important for the mediation of short-range Wg signaling [28]. By contrast,

loss of flotillin, which reduces Wg carriers (identified to be distinct from exosomes based on their density on a sucrose gradient), specifically affect the expression range of Distalless (Dll) (a long-range target) without affecting the expression of Sens [44], suggesting that there may be at least two types of carrier with different functional consequences.

Similarly, downregulation of Shrub (ESCRT-III) in Hh-producing cells can perturb the endogenous-Hh gradient and reduce the expression of its long-range target gene Dpp. However, no effect on the expression of short [Patched (Ptc)] or intermediate (Col) target genes was seen [26,32]. Flotillin-based carriers [44] are also involved in mediating long-range signaling of Hh without affecting its short-range target genes. Although flotillins are present in exovesicular fractions [30] and are independently known to regulate exovesicular secretion [77], their role in regulating Hh and Wg release is unclear. It remains to be seen whether Flotillin-based carriers regulate Wg and/or Hh exosome production, influence the composition of exosomes and hence their range/functional abilities, or represent a distinct form. Studies appear to highlight an important difference between the roles of Wg and Hh exosomes; unlike Wg, exosomal Hh is involved in long-range signaling but not for short or intermediate target gene activation (Figure 3).

Besides exosomes, Lpps mediate long-range signaling in wing discs. Ubiquitous expression of Lpp RNAi resulted in extracellular accumulation close to the source, suggesting that Lpp-based carriers could mediate long-range Wg and Hh transport. While the range of Dpp was reduced on Lpp RNAi, expression of Patched and Collier, which are short- and intermediate-range targets of Hh signaling, remained unaffected. Similarly, Dll expression was affected on Lpp RNAi but its effects on Sens expression were not tested [34].

Perturbations of proteins involved in the regulation of actin structure and dynamics, such as Capping protein, SCAR, and Pico affects the length of cytonemes that emanate from Hh-producing cells as well as the long-range spread of Hh and target gene activation [35]. Thus, to date, the forms of Hh that mediate its short-range signaling have not been identified. As observed for Wg, it is possible that exosomes could also be employed for signaling locally. The inability to identify the players involved in short-range Hh signaling might be due to inefficient depletion of the molecular players involved and/or compensation by other molecular players or extracellular forms.

Alternatively, soluble forms of Hh may facilitate short-range Hh signaling, because the MMP-derived lipid-free form of Hh has not been identified *in vivo* and the mechanism of generation of HhN* has not been identified. The signaling ability and range of endogenous soluble forms of Hh remain to be evaluated. Thus, while various forms of Hh have been identified and significant work has been done to evaluate their roles, it is not entirely clear whether these have differential signaling abilities or ranges. Several functional aspects of these different forms remain unclear (see Outstanding Questions).

Compositional Heterogeneity of Hh and Wg Carriers

Exosomes also carry other functional signaling proteins and regulatory RNA molecules. Whether the exosomal form of morphogen release is also involved in the targeted delivery of other signaling molecules for synergistic signaling and cell-fate determination remains an

exciting possibility to probe [52]. A recent study demonstrates that, besides target gene activation, interaction of Hh with Boc is also required for the activation of JNK and ABL kinases to facilitate neuronal differentiation and neurite growth [78]. Here it remains to be seen whether a specific form of extracellular Hh is involved in the process. It was demonstrated recently that Smo activation requires membrane cholesterol [79,80]. The cholesterol-sensing cysteine-rich domain of Smo is present extracellularly [81]; could this domain be involved in sensing the lipids in Hh carriers as well?

Disease Implications of the Various Forms of Hh and Wg

Hh and Wnt signaling is activated in several cancers [19]. Some Hh/Wnt ligand-dependent cancers exhibit increased autocrine, paracrine (tumor cells to stroma), or reverse paracrine (stroma to tumor cells) signaling abilities [82,83]. Hh and Wnt signaling are implicated in tumor progression and metastasis, relapse, and drug resistance. The focus so far has been on identifying the signaling pathways responsible for cancer progression as well as on evaluating the efficacy of various signaling inhibitors as druggable targets [82–85]. However, despite the encouraging preclinical data [82–85], it remains unclear whether a signaling inhibitor drug can be made efficacious with acceptable toxicity and without compromising the signaling's role during tissue homeostasis and repair. The identification of extracellular forms and/or partners of Hh and Wg that are important for disease progression will be extremely valuable not only in improving our understanding of morphogens in cancer but also in identifying effective drug targets.

Concluding Remarks

Morphogens are reported to be secreted in more than one form. There is compelling evidence supporting the release of Hh and Wg on exosomes [26,28,29,31,32] and lipoproteins [33,34,66]. However, given that Hh can also be secreted in a soluble/sterol-free signaling-competent form, it remains to be resolved whether exosomes or lipoproteins have roles other than activating Hh target genes. Exosomes contain lipids as well as many different proteins and cellular RNAs [30,86]. Do these constituents contribute to the cell-fate determination of target cells? Like Hh and Wg proteins, even secreted morphogens such as vascular endothelial growth factor (VEGF), FGF, EGF, and Dpp are present on exosomes [87] and/or are ferried via cytonemes [74]. Do these different morphogen carriers provide unique abilities to these morphogens? While it is tempting to hypothesize that different secreted forms of morphogens perform non-overlapping roles, compelling evidence for this hypothesis requires definition of the proficiency of the various forms of extracellular morphogens and their precise mechanisms of formation.

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Box 1**Mechanisms of Exovesicle Biogenesis**

Conventionally, MVB biogenesis requires components of the ESCRT machinery. In brief, ESCRTs comprise four distinct protein complexes (i.e., ESCRT-0, -I, -II, and -III) that act in a sequential manner to generate ILVs by budding and scission of the MVB-limiting membrane [48]. HGS and signal transducing adaptor molecule (STAM) 1 and 2 form the ESCRT-0 complex. HGS loads onto endosomes via an interaction between its FYVE domain and endosomal lipid phosphatidylinositol 3-phosphates [88]. HGS and STAM are known to be critical in sorting ubiquitinated cargoes, via their ubiquitin-recognition domains, into clathrin-coated microdomains [89,90]. Loading of ESCRT-0, leads to a cascade of events to recruit the subsequent ESCRTs (i.e., -I, -II, and -III) [48]. Loading of the ESCRT-III component Shrub/Vps32 is important in generating membrane curvature and hence ILV formation [91]. ESCRTs are further assisted by accessory proteins such as Alix and Vps4 in ILV formation [48]. Vps4, an AAA-ATPase, is necessary for the dissociation of ESCRT-III assemblies from the endosome for subsequent cycles [92]. Alix can potentially link ESCRT-I and ESCRT-III subunits, contribute to ubiquitin-dependent and -independent modes of cargo sorting, and aid in ILV formation by interactions with ESCRT-III components [93]. Besides ubiquitinated proteins, several non-ubiquitinated proteins have also been identified in exosomes. However, unlike ubiquitinated proteins, much less is known about the mechanisms for sorting non-ubiquitinated proteins into exosomes. Several players have been implicated in this process. It has been demonstrated that non-ubiquitinated proteolipid protein (PLP) is secreted on exosomes by an oligodendroglial precursor cell line in an ESCRT-independent, ceramide-dependent manner [94]. Tetraspanins have also been implicated in the regulation of exosomal secretion of proteins such as *b*-catenin [95] and premelanosome protein (PMEL) [96]. It has been proposed that tetraspanins can interact with each other as well as with several other cellular proteins (e.g., integrins, ICAM-1, VCAM-1) to form domains/webs that can assist the sorting of proteins into ILVs and hence exosomes [97,98]. Similarly, syndecan clustering along with syntenin–Alix interactions has also been implicated in the sorting of proteins into exosomes [99,100] (Figure I).

Microvesicles are generated by outward budding of the PM. Microvesicles are mainly reported for their secretion of viruses. Microvesicle biogenesis is known to involve ESCRT-I, -II, and -III and Vps4 function [57].

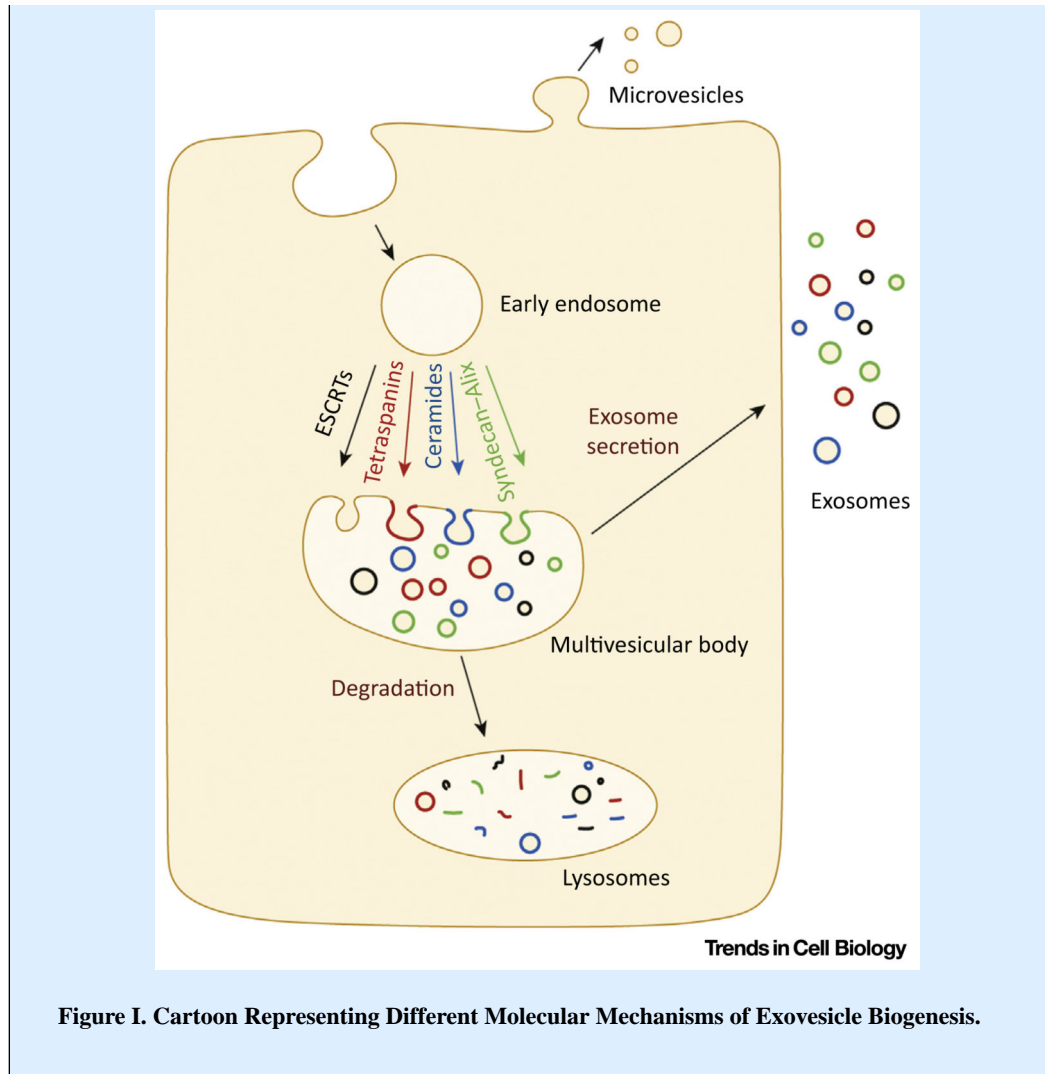


Figure I. Cartoon Representing Different Molecular Mechanisms of Exovesicle Biogenesis.

Trends

The membrane-anchored morphogens Hedgehog (Hh) and Wingless (Wg) play major roles in tissue patterning, tissue maintenance, and disease progression.

Hh and Wg must be secreted for their paracrine signaling function. In addition to being secreted on exovesicles, Hh and Wg are found on circulating lipoproteins and decorating cytonemes as well as in soluble forms.

The mechanism of Hh exovesicle generation involves its endocytosis from the cell surface regulated by Shibire (*Drosophila* dynamin) and Rab5-mediated endosomal progression, followed by sorting to intraluminal vesicles (ILVs) located in multivesicular bodies (MVBs) in an endosomal sorting complex required for transport (ESCRT)-dependent manner and subsequent release on exovesicles.

In addition to the morphogens, exovesicles carry other targeting and regulatory molecules including cellular RNAs. Besides activating their direct downstream signaling targets, exosomes can deliver cargo that may influence the cell-fate decisions ascribed to these morphogens.

Outstanding Questions

Do different carriers of Hh and Wg represent tissue-specific mechanisms for their release and/or do they have different targets?

Do the accompanying constituents of exosomes that carry morphogens influence their signaling range or efficiency? Are the different morphogens involved in selecting at least some of these partners?

Do the extracellular forms and/or partners of morphogens change under normal versus disease conditions?

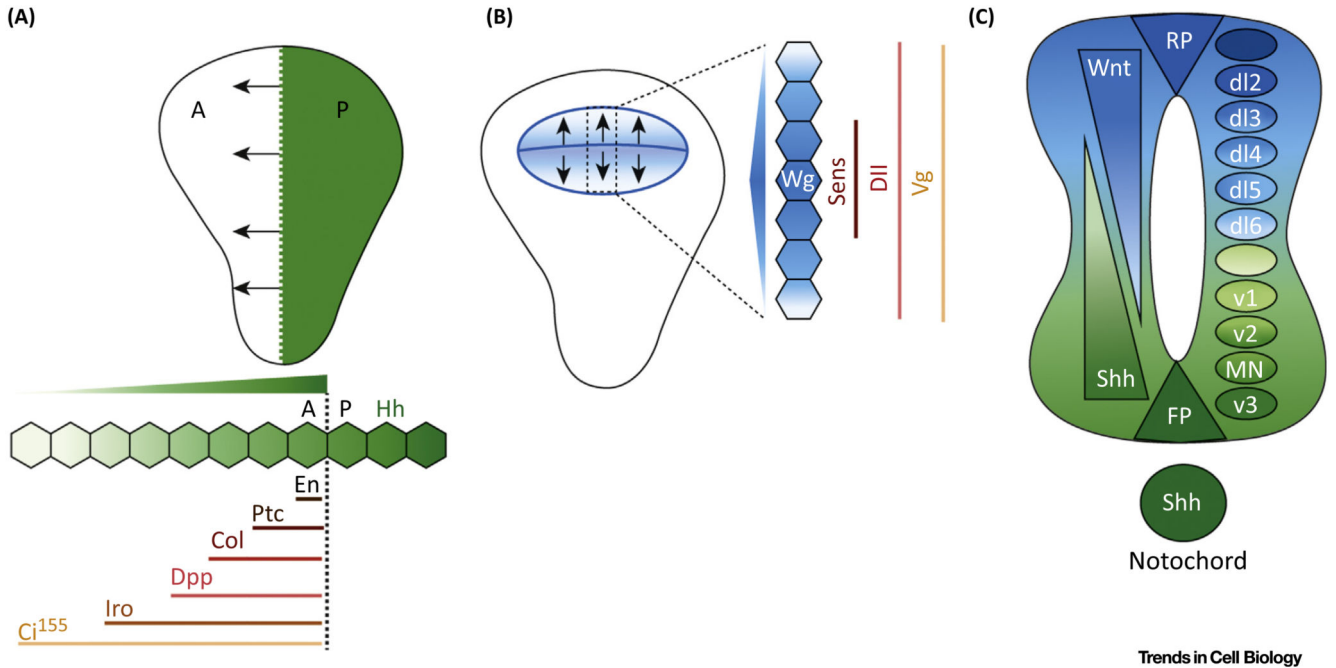
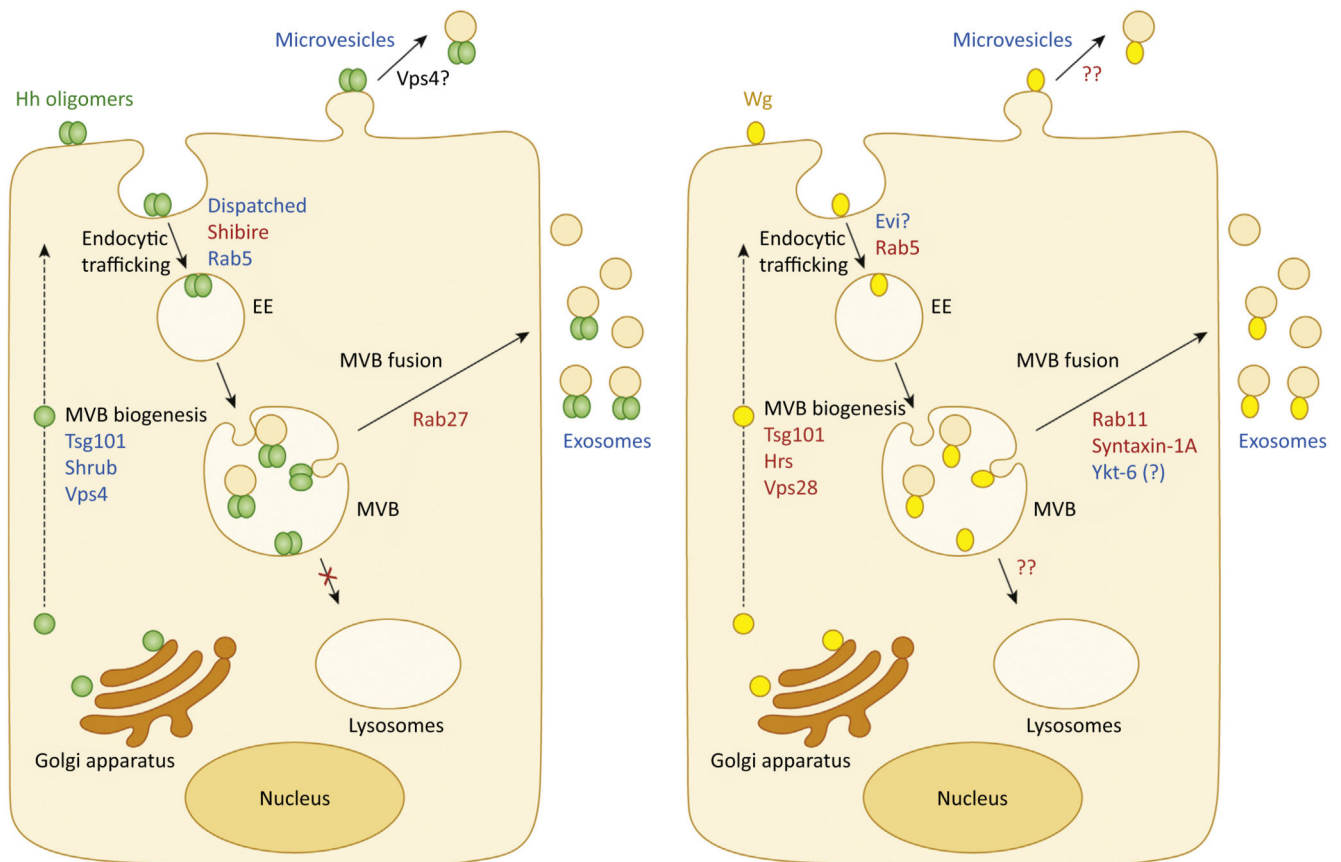


Figure 1. Hedgehog (Hh) and Wnt Signaling in the *Drosophila* Wing Disc and Vertebrate Neural Tube.

(A) Cartoon representing Hh signaling in wing discs. Hh is produced in the posterior domain (green; P) and generates a concentration gradient (arrows) along the anterior domain (white; A). Engrailed (En) and Patched (Ptc) are short-range targets of Hh, expressed by up to three or four cells abutting the AP boundary. Collier (Col) requires intermediate Hh levels, whereas Decapentaplegic (Dpp) and Iroquois (Iro) are long-range targets, expressed ~10–15 cells away along the AP boundary. Stabilization of cubitus interruptus (Ci¹⁵⁵) requires the lowest levels of Hh. Higher levels of this protein are seen near the AP boundary, while cells away from the AP boundary see lower levels of stabilized Ci. (B) Cartoon representing Wingless (Wg) signaling in wing discs. Wg expression is seen at the dorsoventral (DV) boundary in a narrow strip of cells and at the boundary of the wing pouch (blue). Wg forms an extracellular gradient on either side of the DV boundary (arrows) and activates targets in a concentration-dependent manner. Senseless (Sens) requires high Wg levels and Distalless (Dll) intermediate levels with graded expression, while Vestigial (Vg) is a lowest-threshold target. (C) Sonic hedgehog (Shh) (green) and Wnt (blue) during vertebrate neural tube patterning. These emanate from two sources: dorsally (BMPs/Wnts from the epidermis) and ventrally (Shh from the notochord). The roof plate (RP) and floor plate (FP) form the secondary signaling centers, which also secrete BMP/Wnt and Shh, respectively. They form reciprocal gradients to specify specific neural progenitors. Wnt/BMPs specify the dorsal sensory neurons (dI1–dI5) while Shh specifies the ventral motor neurons; highest to lowest: v3, MN, v2, v1, and v0.

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Figure 2. Molecular Players Involved in Exovesicular Release of Hedgehog (Hh) and Wingless (Wg).

Hh (in green) and Wg (in yellow) are secreted from the Golgi apparatus and anchored on the cell membrane via their lipid modifications. Exosomal release requires endocytosis of Hh (via Shibire, Dispatched) and Wg (maybe mediated by Evi; precise role unclear) with sorting in Rab5-containing early endosomes (EEs). From EEs, Hh and Wg are sorted onto intraluminal vesicles (ILVs) by inward budding of multivesicular body (MVB)-limiting membrane. The endosomal sorting complex required for transport (ESCRT) machinery is required for release of Sonic hedgehog (Shh) (Tsg101, Shrub, and Vps4) and Wg (Hrs, Tsg101, and Vps28) on exosomes. Fusion of the MVB with the cell surface results in the release of ILVs in the form of exosomes: Rab27 for Hh, while for Wg, Rab11, syntaxin-1A, and Ykt-6 are thought to be involved in MVB fusion. Endocytosed Hh fails to populate tubular lysosomes, which are known for their role in the degradation of endocytic cargo; in the case of Wg, this possibility has not been probed (marked ??). Besides exosomes, Hh and Wg can also be secreted on microvesicles by plasma membrane budding. In the case of Hh, cell-surface accumulation of Hh on the expression of a dominant-negative form of Vps4 has led to the proposition of microvesicle-mediated Hh secretion; however, for Wg exovesicles this mode of secretion has not yet been proposed (marked ??). Molecular players involved in exovesicular secretion and identified *in vitro* and *in vivo/in vivo*, are highlighted in blue text; molecules identified in specific cell types or in cell-based assays only are in red text.

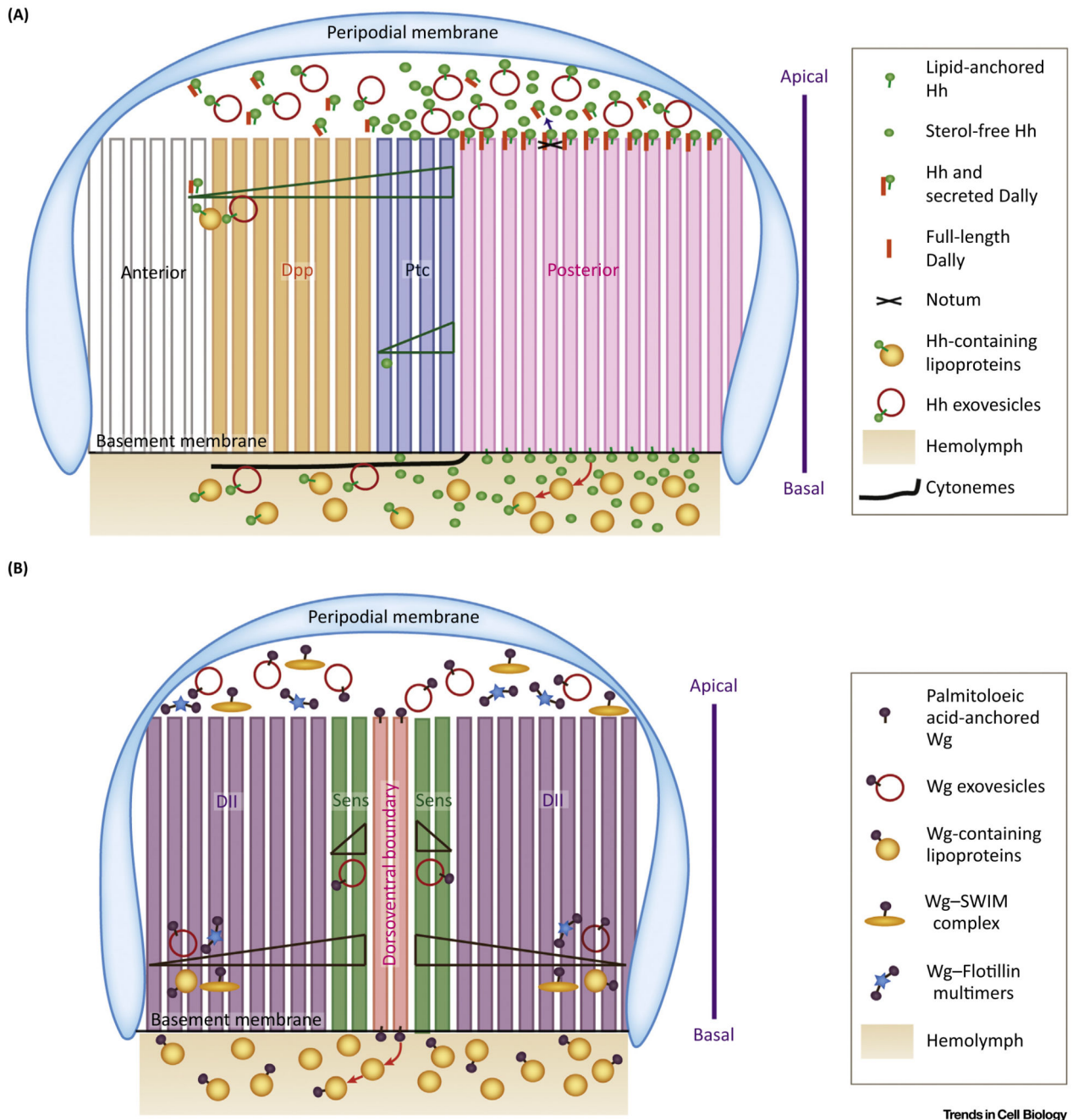


Figure 3. Transport of Morphogens Across Tissue: Multiplicity of Carriers.

(A) Cartoon representing the various extracellular forms of Hedgehog (Hh) and their polarized transport. Hh secretion and transport are seen on both the apical and the basolateral side of the *Drosophila* wing imaginal disc. On the apical side, Hh is secreted on exovesicles in the lumen, which is necessary for its long-range transport. Additionally, a carrier involving Hh and Dally (cleaved by Notum) is also secreted in the apical lumen and is necessary for long-range signaling by Hh. Basally, Hh-containing exovesicles are seen predominantly on cytonemes, which are filopodial structures. The hemolymph is in contact

with the wing imaginal disc on the basal side. Hemolymph contains circulating lipoprotein particles. Hh interacts with lipoproteins, which in turn act as carriers for long-range transport. The identity of a short-range carrier involved in Hh signaling remains elusive and we speculate that the soluble forms of Hh could be crucial for transporting Hh at short distances to activate high-threshold targets like Patched (Ptc). (B) Cartoon representing the various extracellular forms of Wingless (Wg). Wg is also ferried by multiple carriers to facilitate its long-range spread. Secreted complexes of Wg with Flotillin and Swim are necessary for its long-range transport. Wg is also transported basolaterally on lipoprotein particles, which help it to spread over long distances. Exovesicles carrying Wg are necessary for its short- as well as long-range transport, thus differentiating them from exovesicles, which are uniquely employed only for long-range Hh spread.