EMBO
reports

review

Regulation of Wnt protein secretion and its role in gradient formation

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In metazoans, many developmental and disease-related processes are mediated by Wnt proteins, which are secreted by specific cells to regulate cellular programmes in the surrounding tissue. Although the Wnt-induced signal-transduction cascades are well studied, little is known about how Wnts are secreted. The discovery of Porcupine, an endoplasmic-reticulum-resident acyltransferase, led to closer inspection of the secretory routes of Wnts, and the analysis of Wnt secretion has become an exciting new area of research. Wnt post-translational modifications, interaction partners and subcellular localizations now indicate that Wnt release is tightly regulated. In this review, we summarize recent advances in the field of Wnt secretion and discuss the possibility that separate pathways might regulate the release of lipid-linked morphogens for short-range and long-range signalling.

Keywords: acylation; gradient; morphogen; secretion; Wnt
EMBO *reports* (2008) **9**, 977–982. doi:10.1038/embor.2008.167

See Glossary for abbreviations used in this article.

Introduction

Many developmental communication processes are mediated by morphogens, which are secreted signalling molecules that provide positional information for cells surrounding the producing 'organizer' cells. One of the best-studied morphogen families is the Wnt protein family. Wnts are essential regulators of developmental decisions and of homeostatic processes, such as those in gut epithelia. Aberrant regulation of Wnts and their signal-transduction cascades have been linked to the formation of various human cancers (reviewed in Clevers, 2006; Klaus & Birchmeier, 2008).

Despite the importance of Wnts for the life of probably all metazoan organisms, it remains largely unknown which molecular mechanisms control their secretion, why Wnt secretion requires tight regulation and whether this is a property of other, if not all, secreted signalling molecules. In this review, we focus on the molecules and mechanisms that are known to be involved in Wnt secretion. We discuss how these different components might interact to control Wnt release, and speculate about whether secretion of Wnts might be a representative mechanism for the release of other signalling molecules.

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**Submitted 19 May 2008; accepted 24 July 2008;
published online 12 September 2008**

Wnt gradient and the 'hydrophobicity problem'

The formation of morphogen concentration gradients might require freely diffusible proteins that can traverse the extracellular space. However, in contrast to predictions made based on their primary amino-acid sequence, experimentation showed that Wnt proteins are hydrophobic and probably membrane-associated, due to acylation by palmitoyl and palmitoleoyl groups (Takada *et al*, 2006; Willert *et al*, 2003; Zhai *et al*, 2004). The enzyme most likely to be responsible for lipid modifications of Wnts is the endoplasmic-reticulum-resident acyltransferase Porcupine (Porc; Kadowaki *et al*, 1996; Takada *et al*, 2006; Zhai *et al*, 2004). Loss of Porc function reduces hydrophobicity and membrane association of Wingless (Wg), which is the *Drosophila* homologue of human WNT1 (Zhai *et al*, 2004), and causes the accumulation of Wg in the endoplasmic reticulum (van den Heuvel *et al*, 1993).

Since the discovery of Wnt lipid modifications, researchers have tried to elucidate how a hydrophobic protein can be transported long distances through tissues. Acylated Wnt is likely to accumulate in the proximity of secreting cells, where it is able to induce the activation of short-range target genes. This 'stickiness' might even be required for accumulating sufficiently high amounts of Wnts in order to perform short-range signalling. However, membrane association would not allow free diffusion through the extracellular space nor the activation of target genes ≥20 cell diameters from the secreting cells (Zecca *et al*, 1996).

One model for long-range distribution could be that Wnts form multimeric complexes, with the lipid chains facing the interior of a 'Wnt micelle'. This form of aggregation through a palmitoyl anchor has been implicated in the extracellular movement of Hedgehog (Hh; Chen *et al*, 2004; Zeng *et al*, 2001), another hydrophobic morphogen (Pepinsky *et al*, 1998; Porter *et al*, 1996).

Also, lipoprotein particles (LPPs) might be a mode of lipid-linked morphogen movement. LPPs colocalize with Wg and, in this context, were first termed 'argosomes' (Greco *et al*, 2001). Their rate of spreading is similar to the speed of Wg gradient formation across the wing disc, suggesting that Wg might travel on argosomes through the tissue (Greco *et al*, 2001). On the basis of their observations, Greco and

Glossary

AP-2	adaptor protein complex AP-2
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
HEK293T	human embryonic kidney 293T cell line
HSPG	heparan sulphate proteoglycan
LDL	low-density lipoprotein
Vps	vacuolar protein sorting

colleagues proposed that argosomes are products of Wg-producing cells. However, in a subsequent study, Panakova and colleagues showed that argosomes are exogenously derived LPPs, which are present in endocytic compartments in the secreting cell and in the extracellular space (Panakova *et al*, 2005). They provided evidence that Wg is associated with lipophorin, the *Drosophila* lipoprotein, in the extracellular space, and that loss of lipophorin reduces the range of Wg signalling.

LPPs consist of a lipid monolayer that harbours apolipoproteins, surrounding a complex of cholesterol and triacylglycerides, and they enter cells through LDL-receptor-mediated endocytosis (reviewed in Rodenburg & Van der Horst, 2005). In *Drosophila*, LPPs are produced in the fat body and might act as lipid cargo carriers through the haemolymph (Kutty *et al*, 1996). It was recently reported that membrane-linked HSPGs recruit lipophorin to wing-disc cells (Eugster *et al*, 2007). Lipid-modified Wnts might therefore move over long distances by the immersion of the hydrophobic anchor in an LPP, and might subsequently be recruited by receiving cells through their cell-surface HSPGs and/or LDL receptors.

It is likely that the distribution of Wnt proteins on LPPs requires a process in the secreting cell that loads lipid-linked morphogens onto LPPs. Wg has been detected in lipid rafts, a phenomenon that depends on its lipid modifications, which are probably mediated by Porc (Zhai *et al*, 2004), and in endocytic vesicles in Wg-producing cells of *Drosophila* embryos (Pfeiffer *et al*, 2002). How could lipid modifications, endocytosis and the presence of Wnts in membrane microdomains be integrated with LPPs? One model could be that Wnts are secreted but the hydrophobic anchor prevents their release from the extracellular leaflet of the plasma membrane. Endocytosis from lipid rafts might deliver the ligands to endocytic compartments in which they could associate with LPPs through their lipid anchor. However, the presence of Wnts in endocytic vesicles might be a result of ligand–receptor interaction during signalling (Piddini *et al*, 2005) and might not be associated with secretion.

Regulation of Wnt secretion by Evi/Wls/Sprinter

Recently, a novel regulator of Wnt secretion was identified in *Drosophila* by independent groups. Evenness interrupted (Evi; also known as Wntless (Wls) and Sprinter) is a multispan transmembrane protein (Fig 1A) that is specifically required for Wnt secretion but not for the secretion of other morphogens (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006). Evi localizes to components of the secretory pathway, especially the Golgi (Banziger *et al*, 2006; Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008) and the plasma membrane (Bartscherer *et al*, 2006; Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008; Fig 1B,C). In addition, Evi colocalizes with endosomal markers, indicating its presence in endosomes (Belenkaya *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). Localization in these compartments points towards a function of Evi downstream from the endoplasmic-reticulum-resident protein Porc in the secretory pathway. Recently, Port and colleagues showed that Wg accumulates in the Golgi of *evi*-deficient producing cells in wing imaginal discs, which is a strong argument for an essential role of Evi in the Golgi and also downstream from it (Port *et al*, 2008).

What role could Evi have in the Golgi of producing cells? Evi does not contain any conserved domains that could illustrate its molecular function apart from a putative signal sequence and several transmembrane domains (Fig 1A). Several conserved cysteines might be involved in the proper folding of Evi or in the intermolecular formation of disulphide bridges. An interaction between Evi and Wnts has been predicted on the basis of colocalization and co-immunoprecipitation experiments (Banziger *et al*, 2006).

The interaction between Evi and Wnt, and their localization to both the Golgi and the plasma membrane, imply that they associate in the Golgi where they are incorporated into vesicles and transported to the plasma membrane. In polarized *Drosophila* follicle cells, an Evi–EGFP fusion protein is enriched at apical and lateral membranes, whereas it is largely absent from the basal surface (Fig 1C). In addition, in *evi*-deficient Wg-producing cells of wing imaginal discs, the apical localization of Wg is lost (Bartscherer *et al*, 2006). One possible explanation is that Evi acts as a cargo receptor, assisting Wnt/Wg to reach the apical side of producing cells, from where it is released. So far, the molecular function of Evi remains unknown.

Evi/Wls/Sprinter recycling by the retromer complex

The requirement of another Wnt regulator in producing cells was recently discovered (Coudreuse *et al*, 2006; Prasad & Clark, 2006). Retromer is a conserved multiprotein complex composed of members of the Vps family that is involved in the selective retrieval of cargo receptors from endosomes to the Golgi. In the absence of retromer, cargo receptors accumulate in endosomal structures and are subsequently degraded in lysosomes (Seaman, 2005). Coudreuse and colleagues observed that mutations in components of the retromer complex in *Caenorhabditis elegans* reduce EGL-20/Wnt target gene expression and affect the formation of the EGL-20/Wnt gradient. The authors proposed that retromer acts in a secretion route that is dedicated to the release of a subset of Wnts that is packaged for long-range signalling (Coudreuse *et al*, 2006). However, it was subsequently shown that retromer is involved not only in long-range but also in short-range signalling, and that Wnts are not secreted in the absence of retromer. Loss of retromer components, such as Vps35, causes accumulation of Wg inside Wg-producing cells, accompanied by reduced amounts of extracellular Wg (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Port *et al*, 2008). Even though differences in the requirement of retromer for Wnt secretion might exist between different organisms, these phenotypes resemble *evi* loss-of-function phenotypes, and suggest that retromer and Evi might act together to facilitate Wnt secretion. Several lines of evidence indicate that retromer stabilizes Evi protein levels by preventing it from being degraded in lysosomes (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008; Pan *et al*, 2008; Port *et al*, 2008; Yang *et al*, 2008). As Evi has been detected at the plasma membrane as well as in endocytic vesicles, and the retromer complex has been implicated in the retrieval of proteins from endosomal compartments to the *trans*-Golgi network, it is likely that retromer supports Wnt secretion by transporting Evi

back to the Golgi after its internalization from the plasma membrane. An interaction between Vps35 and Evi has been proposed based on co-immunoprecipitation experiments, and recycling of Evi was shown with antibody-uptake assays (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008). The internalization of Evi depends on clathrin-mediated endocytosis in *C. elegans* (Pan *et al*, 2008; Yang *et al*, 2008) and in *Drosophila*. In wing imaginal discs, RNA interference against AP-2 reduces the amount of Evi in the Golgi and leads to the accumulation of Wg in Wg-producing cells (Port *et al*, 2008). Similarly, Evi accumulates at the plasma membrane in *shibire* (*shi*)-mutant tissue (Belenkaya *et al*, 2008). Shibire is the *Drosophila* dynamin, and is a large GTPase involved in early endocytic events that has been implicated in Wg secretion (Strigini & Cohen, 2000). Together, these data suggest that clathrin-mediated endocytosis precedes Golgi-retrieval of Evi by retromer, and that endocytosis and recycling of Evi is an essential process for the secretion of Wnt proteins.

Different secretion routes for short-and long-range signals?

Several factors involved in Wnt secretion and gradient formation affect long-range rather than short-range signalling, raising the question of whether two different secretory mechanisms exist that release Wnts for short-range or long-range signalling. One of these proteins is the microdomain-associated protein Reggie-1 (also known as Flotillin-2; Hoehne *et al*, 2005; Katanaev *et al*, 2008). Loss of *reggie-1* in *Drosophila* causes wing-margin defects and narrows the Wg concentration gradient. On overexpression of *reggie-1*, activation of the long-range target gene *distal-less* (*dll*) is increased, to the detriment of the short-range target *senseless* (*sens*). Signal transduction through Hh seems to be affected in a similar way (Katanaev *et al*, 2008).

Similar effects have also been observed while studying LPPs. Both Wg and Hh seem to interact with these structures in *Drosophila* wing imaginal discs (Panakova *et al*, 2005). RNA interference against lipophorin does not compromise the Hh short-range target gene *collier* (*col*), but reduces the range of expression of *decapentaplegic* (*dpp*), a long-range Hh target gene. In addition, Hh and Wg gradients are reduced, implying that LPPs promote long-range signalling of lipid-linked morphogens.

Separate secretion pathways might, therefore, regulate the release of differentially packaged morphogens for short-range and long-range signalling. As Reggie-1 and LPPs both affect long-range rather than short-range signalling, they might act in the same pathway destined for the release of highly diffusible forms of Hhs and Wnts. Do Reggie-1 and LPPs also act in the same Wnt-secretion pathway as Evi and retromer? Because Wg accumulates in the Golgi of *evi*-mutant cells (Port *et al*, 2008), and the Golgi is a central component of the secretory pathway, both short-range and long-range signalling should be affected by loss-of-function of Evi. However, although the induction of the short-range target gene *sens* is markedly reduced, the expression of the long-range target gene *dll* is barely changed (Banziger *et al*, 2006; Bartscherer *et al*, 2006). A similar phenomenon is seen for the retromer complex (Belenkaya *et al*, 2008; Franch-Marro *et al*, 2008). Could this mean that Evi and the retromer complex are primarily required for the release of Wnts that are dedicated to short-range signalling? Although this is a possibility, *evi* is maternally inherited in *Drosophila* (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006), and so residual Evi might rescue the expression of low-threshold target genes. Another argument against a single secretion pathway for short-range and long-range Wg signalling is the fact that Hh and Wg share

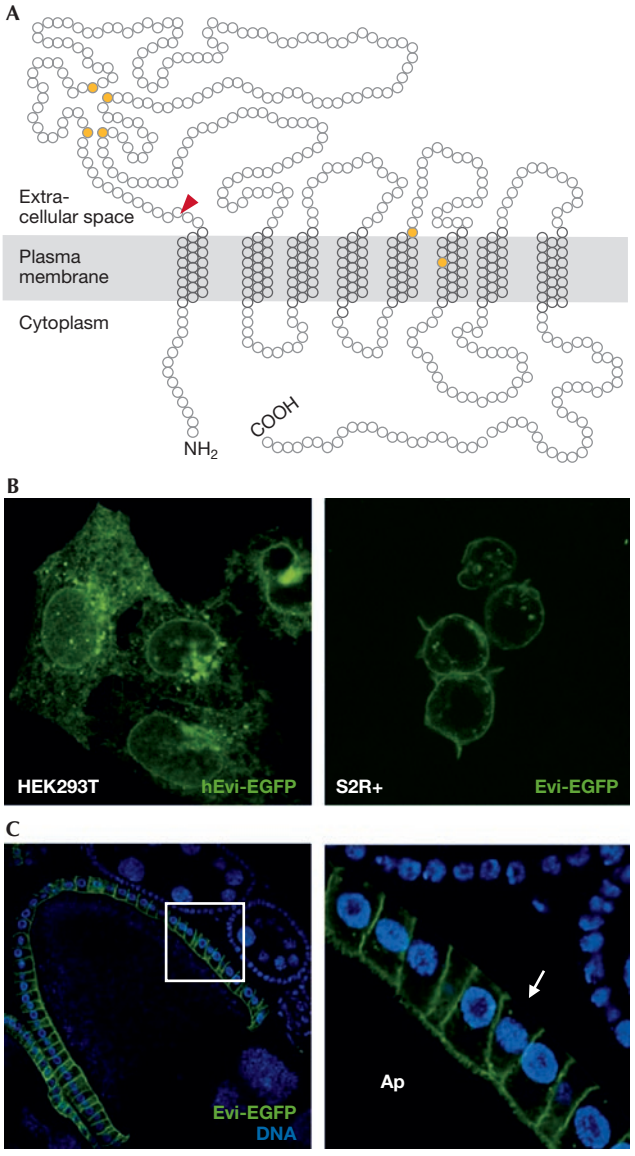

Fig 1 | Putative structure and localization of Evi/Wls/Sprinter. (**A**) Possible topology of Evi. Conserved cysteine residues (yellow) and a putative signal peptide cleavage site (red arrowhead) are indicated. (**B**) Human Evi–EGFP or *Drosophila* Evi–EGFP fusion proteins (green) localize to membranes in unpolarized human HEK293T or *Drosophila* S2R$^+$ cells, respectively. (**C**) In polarized *Drosophila* follicle cells, Evi–EGFP expressed under the control of the tubulin promoter, is concentrated at the apical (Ap) side, but is largely absent from the basal side (arrow). A confocal section through an egg chamber is shown. The follicle cells surround the oocyte with their apical side facing the centre of the egg chamber. DNA is shown in blue. Evi, Evenness interrupted; Wls, Wntless.

Reggie-1 and lipophorin particles as components of their secretory machinery (Katanaev *et al*, 2008; Panakova *et al*, 2005), whereas Evi and retromer seem to be specific for Wg (Banziger *et al*, 2006; Bartscherer *et al*, 2006; Goodman *et al*, 2006). Accordingly, the sterol-sensing protein Dispatched (Disp) seems to be specifically required for the release of cholesterol-modified Hh (Burke *et al*,

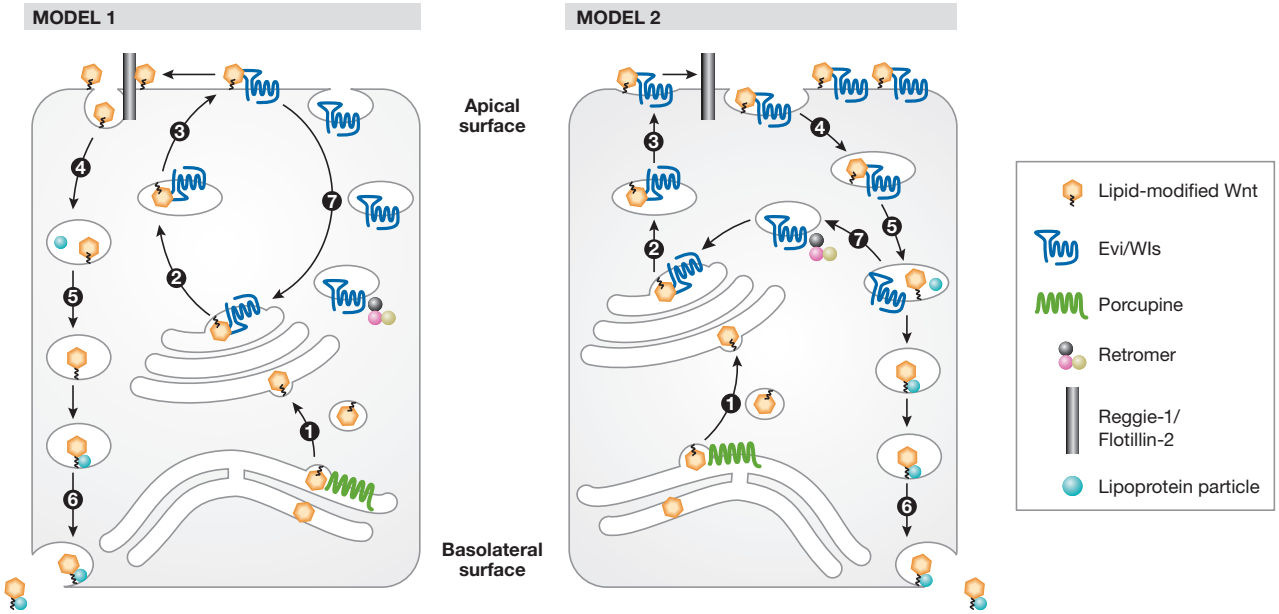

Fig 2 | Models of Evi/Wls/Sprinter-dependent Wnt secretion. Wnt is lipid-modified in the endoplasmic reticulum by Porc (step 1) and travels to the Golgi where it binds to Evi (step 2), facilitating its delivery to the apical plasma membrane (step 3). In model 1, Wnt and Evi dissociate on the plasma membrane. Wnt associates with Reggie-1/Flotillin-2 containing microdomains, and is internalized (step 4). In endosomal compartments, Wnt is loaded on lipoprotein particles by an unknown mechansim (step 5), and is released from the basolateral surface for long-range signalling (step 6). In model 2, Wnt and Evi are internalized together (step 4), and dissociate in endosomal compartments (step 5). There, Wnt is loaded on lipoprotein particles and released from the basolateral surface (step 6). In both models, Evi is recycled to the Golgi in a retromer-dependent manner (step 7). Note that, in both models, membrane-bound Wnt signals from the apical membrane to induce short-range targets, whereas the long-range concentration gradient forms on the basal surface. Evi, Evenness interrupted; Porc, Porcupine; Wls,Wntless.

1999; Kawakami *et al*, 2002). This suggests that a mechanism for long-range secretion is common to both Hh and Wg proteins, and possibly for other lipid-linked morphogens, whereas secretion for short-range signalling is regulated by different molecular mechanisms.

During the secretion of Wnts, the two putative pathways might both depend on Evi in the beginning, but branch downstream from its action (Fig 2). One model could be that Evi guides membrane-anchored Wnts from the endoplasmic reticulum or the Golgi to the apical plasma membrane, where they might induce short-range target-gene expression in neighbouring cells. Association with Reggie-1-containing microdomains might render Wg susceptible for endocytosis. This is consistent with the study by Pfeiffer and colleagues showing that Wg produced by expressing cells is endocytosed by the same cells (Pfeiffer *et al*, 2002), and a more recent study by Gallet and colleagues indicating that endocytosis occurs from the apical surface of these cells (Gallet *et al*, 2008). Endocytosis from the plasma membrane might then deliver Wnts to endosomal compartments in which they encounter internalized LPPs from the haemolymph. Lipophorin particles have been detected in the same endosomal compartments as Wg (Panakova *et al*, 2005). We can only speculate as to which, if any, proteins might facilitate the loading of Wnts on LPPs. However, Wnt-loaded LPPs might then be released, possibly from the basolateral cell surface, to mediate long-range signalling. Greco and colleagues suggested that argosomes/LPPs bud off from basolateral membranes (Greco *et al*, 2001). In addition, extracellular detection of Wg in wing imaginal discs revealed a broad Wg gradient across the basolateral surface, whereas a steep and narrow gradient was present at the apical side (Panakova *et al*, 2005; Strigini & Cohen, 2000). Also, intracellular Wg is primarily detected close to the apical plasma membrane (Strigini & Cohen, 2000), and loss of Evi or retromer function disturbs this localization (Bartscherer *et al*, 2006; Franch-Marro *et al*, 2008). It is therefore possible that membrane-associated Wg accumulates on the apical membrane, whereas the mobile pool of Wg, with its lipid anchor buried in an LPP, might be released from the basolateral side, leading to two separate gradients with differences in activity and range.

The events and molecules that facilitate the internalization of Wnts from the apical plasma membrane remain to be elucidated. The glypican Dally-like seems to be involved in this process (Gallet *et al*, 2008). In addition, the lipid-raft-associated Reggie-1 could facilitate internalization of Wnts from lipid rafts. Another candidate could be Evi. Are Wnts completely released from their association with Evi (Banziger *et al*, 2006) at the plasma membrane? Evi endocytosis is crucial for proper Wg secretion in *Drosophila* (Port *et al*, 2008). Because Wnts and Evi are both present in endosomal compartments, it is possible that they are jointly internalized and that Evi not only acts as a cargo receptor that allows Wnts to reach the plasma membrane, but also helps them to reach endosomal compartments. After delivery of the ligand to LPP-containing endosomes, Evi could be recycled back to the Golgi by retromer, ready to assist more Wnt molecules in secretion (Fig 2).

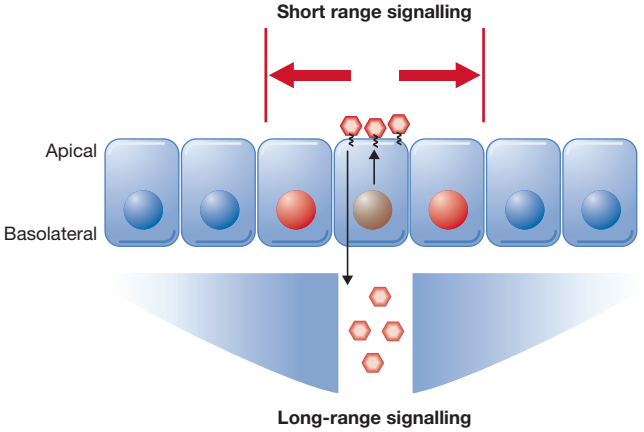

Fig 3 | Two-gradient model. Lipid-linked morphogens are secreted from the apical side of producing cells and accumulate on the surface due to their lipid anchor. There, they activate short-range target genes in adjacent cells (red). Transcytosis delivers them to the basolateral surface where they are secreted, possibly on lipoprotein particles. Diffusion of mobile morphogen complexes leads to the activation of long-range target genes (blue).

Sidebar A | In need of answers

(i) We know that Wnt proteins undergo multiple acylations. Do the lipid moieties mediate membrane tethering? Are they required for ligand–receptor interaction or proper folding of the ligand?

(ii) Several molecules are known to regulate Wnt release. What are the molecular mechanisms of proteins such as Evi/Wls, Reggie-1 and Dally-like in Wnt secretion? Which factors facilitate endocytosis and loading of Wnts on lipoprotein particles?

(iii) Wnt ligands, such as Wingless, have short-range and long-range target genes. Key open questions remain as to how secretion is involved in morphogen-gradient formation and whether different secretion routes exist. Could such mechanisms be common to all morphogens?

Concluding remarks

So far, many of the molecular mechanisms that guide Wnt protein secretion have yet to be resolved (Sidebar A). Why are Wnts lipid-modified and why do they need specialized accessory proteins to reach the surface of producing cells? One possible answer could be that lipid modifications are required to accumulate Wnts near producing cells, in order to reach the threshold concentration that is required for the activation of short-range target genes. One example for such a model is Spitz, the ligand for EGFR in *Drosophila*. Spitz is palmitoylated by the same acyltransferase that palmitoylates Hh, and loss of palmitoylation extends the Spitz gradient, but reduces its concentration around Spitz-producing cells (Miura *et al*, 2006). Lipid modifications might therefore prevent the ligand from diffusing too fast from the site of secretion, facilitating the local concentration and activation of high-threshold target genes.

As lipid modifications render soluble molecules hydrophobic, mechanisms must exist to enable them to signal over many cell diameters in a tissue. One obvious solution would be that only a part of the ligand pool is coupled to lipids; in such a scenario, the other part would be free to diffuse through the extracellular space. However, most Wnts in the extracellular space bear a lipid anchor and are associated with hydrophobic structures or membranes (Panakova *et al*, 2005). Lipid modifications might therefore have co-evolved with auxiliary proteins and mechanisms that facilitate the movement of hydrophobic morphogens through the tissue. A recent study suggests that Evi is not required for the secretion of *Drosophila* WntD, which is a Wnt protein that is not palmitoylated (Ching *et al*, 2008). Evi might therefore be specialized to guide acylated Wnts to the plasma membrane. The observations that Wg accumulates in the Golgi in the absence of Evi (Port *et al*, 2008) and that it cannot reach the extracellular space (Banziger *et al*, 2006; Bartscherer *et al*, 2006) point towards this possibility.

Do other signalling molecules share these mechanisms? Hh might be a good candidate as it is post-translationally modified by a palmitoyl and cholesterol group, and, therefore, similar to Wg, it is probably membrane-associated (Gallet *et al*, 2003; Pepinsky *et al*, 1998; Porter *et al*, 1996). In addition, Hh is likely to be present on detergent-resistant microdomains (Rietveld *et al*, 1999) and LPPs (Panakova *et al*, 2005), and the shape of its gradient seems to depend on Reggie-1 (Katanaev *et al*, 2008). Also, similar to Evi, Disp might facilitate the secretion of cholesterol-modified Hh. Disp is required for the apical targeting of Hh-containing structures (Gallet *et al*, 2003), and, in its absence, Hh accumulates in producing tissue (Burke *et al*, 1999). Disp might therefore act as a cargo receptor, guiding Hh to the plasma membrane.

It will be interesting to see whether more secreted signalling molecules are lipid modified, and whether they require specialized proteins for their secretion. Together, the recent findings for Wnt ligands point towards models whereby the activity of morphogens might be elicited by multiple gradients established by distinct, but possibly interdependent, secretory pathways (Fig 3).

ACKNOWLEDGEMENTS

We thank I. Augustin and T. Buechling for critical reading of the manuscript, and anonymous reviewers for their helpful comments. We apologize to any of our colleagues whose work has not been cited due to space limitations. Research in the laboratory of M.B. is supported by grants from the European Commission, the German Research Council and the European Molecular Biology Organization (EMBO) Young Investigator Programme.

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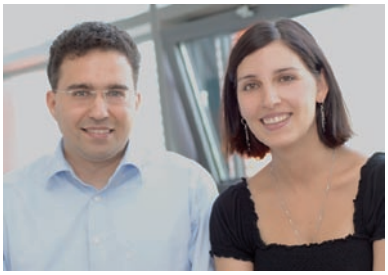

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