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The making of Wnt: new insights into Wnt maturation, sorting and secretion

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In this review, we look at the processes that occur 'behind the scenes' in Wnt signalling, within the Wnt-producing cells. The Wnt community has long been focused upon events that occur downstream of Wnt binding to its receptors, but the recent discovery that the maturation of the Wnt protein may have a profound effect on its signalling properties has excited great interest. In the last 2 years, several key regulators of Wnt production have been discovered, but our global understanding of this process remains relatively poor. Several models that reconcile former and recent observations of Wnt modification, sorting and secretion, and which highlight the potential of this emerging field, are presented here.

Introduction

Wnt glycoproteins are extracellular ligands that can be found in many species, ranging from the sea anemone *Nematostella vectensis* to human (Kusserow et al., 2005). Wnts act as morphogens and control the patterning of the developing embryo by triggering concentration-dependent responses in cells located at a distance from the signal-sending domain (Neumann and Cohen, 1997; Strigini and Cohen, 2000; Zecca et al., 1996). During animal development, Wnt ligands regulate several key processes, such as cell proliferation, cell migration and cell differentiation (Cadigan and Nusse, 1997; Moon et al., 2002; Wodarz and Nusse, 1998). Furthermore, deregulation of the Wnt signalling pathway has been implicated in many pathological disorders, including colon cancer (Bienz and Clevers, 2000; Logan and Nusse, 2004). The intracellular mechanisms that transduce the Wnt signal downstream of its receptors and modulate the expression of its target genes have been extensively studied, and will not be discussed here (for review, see Cadigan and Liu, 2006; Cadigan and Nusse, 1997; He et al., 2004; Moon et al., 2002; van Es et al., 2003) (also see the Wnt Homepage *http://www.stanford.edu/~rnusse/wntwindow.html*). However, little is known about the different steps that control the synthesis and secretion of a functional Wnt protein, and which ensure its delivery to the responding cells along the morphogenetic field. In this review, we focus on Wnt-producing cells and discuss the mechanisms that lead to the production of a fully active Wnt. All known aspects of Wnt maturation will be addressed: from its post-translational modification in the endoplasmic reticulum (ER) to its sorting within the expressing cells and, finally, to its secretion as a long-range-acting protein. Although the influence of Wnt properties on the formation of an extracellular concentration gradient will be mentioned, the multiple mechanisms that regulate the spreading of Wnt will not be presented here. For overviews of these processes, the reader is referred to other reviews (Cadigan, 2002; Eaton, 2006; Strigini and Cohen, 2000; Vincent and Dubois, 2002).

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Structure of Wnts

Except for *Drosophila* Wingless (Wg), Wnt3/5 and Wnt4, which are all larger proteins, the different Wnts generally have similar structural characteristics (Miller, 2002) (Table 1). Thus, most Wnts are approximately 350 amino acids long and have a molecular weight of about 40 kDa. Wnts contain several charged residues and an average of 23 to 25 cysteines, some of which are highly conserved between different species (Fig. 1). Several of these cysteines are involved in inter- and intra-molecular disulfide bonds, participating in Wnt folding and multimerization (Tanaka et al., 2002). Surprisingly, despite the presence of charged residues in their primary sequences, mouse Wnt3A and *Drosophila* Wg were found to be hydrophobic molecules (Willert et al., 2003; Zhai et al., 2004). This hydrophobicity confers a strong affinity for cell membranes, which raises questions as to how these proteins spread in the extracellular space. Wnts do not contain any defined domains, but do have an *N*-terminal hydrophobic signal sequence that targets them to the ER. Finally, further analysis of the Wnt sequences reveals the presence of a number of potential *N*-glycosylation sites, the functional relevance of which will be discussed below (Fig. 1).

Many properties of Wnts, such as their high hydrophobicity despite the presence of charged residues, cannot be understood from this rough analysis of their primary sequence. Further biochemical characterization of maturing Wnt in its producing cells has brought some answers to these conflicting observations, as discussed below.

Post-translational modifications of Wnt in the ER

When they reach the ER, immature Wnts undergo important post-translational modifications. First, the oligosaccharyl transferase complex (OST) rapidly and efficiently attaches *N*-linked oligosaccharide chains to the appropriate residues on the peptide backbone (Asn^{108} and Asn^{414} in Wg) (Tanaka et al., 2002). However, the biological function of Wnt *N*-glycosylation remains unclear. Indeed, mutation of part or all of the potential glycosylation sites of mouse Wnt1 does not affect its function in a cell culture-based assay (Mason et al., 1992). In cell culture, the secretion of many Wnts by their producing cells is inefficient and most newly synthesized Wnt is retained in the ER (Burrus and McMahon, 1995; McMahon and Moon, 1989; St-Arnaud et al., 1989). Within this compartment, Wnt has been found to associate with the immunoglobulin heavy-chain-binding protein BiP, a member of the Hsp70 family of heat-shock proteins (Burrus and McMahon, 1995; Kitajewski et al., 1992). BiP acts as a chaperone for numerous proteins, and allows the further sorting and secretion of mature and functional proteins only (Gething and Sambrook, 1990; Hurtley et al., 1989; Kassenbrock et al., 1988). Thus, BiP can sequester certain misfolded or unassembled proteins within the ER. Inhibition of Wnt *N*-glycosylation does not increase the relative amount of Wnt bound to BiP, suggesting that this does not severely affect Wnt folding and structure (Burrus and McMahon, 1995). Finally, similarly to other secreted proteins, Wnt *N*-glycosylation may be one of the determinant signals for its apical secretion, which may explain the absence of effect when glycosylation is inhibited in a non-polarized

Table 1. The majority of Wnts have similar structural characteristics

Wnt	Length	Molecular weight	Cysteines	N-glycosylation sites	Closest mammalian homologue
Mouse					
Wnt1	370	41	23	4	–
Wnt2	360	40	24	1	–
Wnt2B/13	389	44	26	1	–
Wnt3	355	40	24	2	–
Wnt3A	352	39	25	2	–
Wnt4	351	39	25	2	–
Wnt5A	379	42	24	3	–
Wnt5B	359	40	24	3	–
Wnt6	364	40	25	2	–
Wnt7A	349	39	25	3	–
Wnt7B	349	39	25	3	–
Wnt8A	354	40	23	1	–
Wnt8B	350	39	24	2	–
Wnt9A	365	40	24	1	–
Wnt9B	359	39	25	1	–
Wnt10A	417	47	24	2	–
Wnt10B	389	43	24	2	–
Wnt11	354	39	26	5	–
Wnt16	364	41	26	1	–
Drosophila					
WgA	468	52	25	1	Wnt1
WgB	415	46	23	1	Wnt1
DWnt2	352	40	24	2	Wnt7
DWnt3/5	1004	113	28	11	Wnt5
DWnt4	539	59	24	2	Wnt9
DWnt6	338	38	21	1	Wnt6
DWnt8	309	35	20	0	Wnt8B
DWnt10	374	42	22	0	Wnt10
C. elegans					
MOM-2	361	40	24	1	Wnt5A
EGL-20	393	45	25	2	Wnt16
LIN-44A	348	38	25	0	Wnt7B
LIN-44B	169	19	17	1	/
CWN-1A	372	42	24	2	Wnt4
CWN-1B	332	37	15	2	Wnt4
CWN-2	360	40	25	2	Wnt5B

The indicated lengths refer to the protein sequences. The molecular weight is given in kDa. The potential N-glycosylation sites have been predicted using the NetNGlyc 1.0 algorithm (http://www.cbs.dtu.dk/services/NetNGlyc/). For further information, see the Wnt Homepage (http://www.stanford.edu/~rnusse/wntwindow.html). A solidus (/) indicates that homology with another Wnt cannot be assigned.

cell assay (Mason et al., 1992). However, this hypothesis has not been experimentally proven, and the exact role of *N*-glycosylation in the production and/or activity of Wnt remains to be elucidated.

The recent purification of mouse Wnt3A from cell culture medium identified an additional post-translational modification (Willert et al., 2003). Specifically, a palmitate group is attached to a cysteine located in the *N*-terminal part of the protein (C77) (see Fig. 1). Wg was also found to be lipid-modified (Zhai et al., 2004), and the high conservation of the cysteine involved suggests that this palmitoylation is a common feature of all Wnts. The function of this second modification is also unclear. The hydrophobic lipid group may promote Wnt targeting to the ER membrane and subsequently allow efficient *N*-glycosylation by the membrane-associated OST complex. However, this cannot be the sole function of the palmitate moiety because, in contrast to loss of *N*-glycosylation of Wnt1, the absence of palmitoylation abrogates Wnt3A function in cell culture (Willert et al., 2003). Alternatively, Wnt palmitoylation may protect the modified cysteine from forming a disulfide bond, thereby preventing the aberrant folding of the protein and its retention in the ER by BiP. Palmitate groups are also known to act as intracellular sorting signals (Bijlmakers and Marsh, 2003). Thus, Wnt lipid-modification may allow the proper routing of the protein through different compartments of the Wnt-producing cells, where Wnts acquire their signalling potential. Finally, Wnt3A hydrophobicity has been found to depend largely upon the presence of the palmitate moiety (Willert et al., 2003). Therefore, this lipid-modification may play a role after secretion by targeting Wnt to the membranes of cells along the morphogenetic field. Such a mechanism may control the spatial distribution of Wnt and increase its local concentration at its receptors, allowing efficient signalling. This model is indeed supported by the capacity of a mutant form of Wnt3A that lacks the palmitoylated cysteine (C77A) to activate a Tcf reporter gene when the potential drop in local concentration is compensated for by overexpression (Willert et al., 2003). However, such a model implies that another mechanism exists that allows Wnt to spread away from its production site by partially balancing the hydrophobicity that is provided by the lipid group.

There is some evidence that the protein Porcupine, which resides in the ER and is a member of the membrane-bound *O*-acyltransferase family (MBOAT), has an influence on both of these

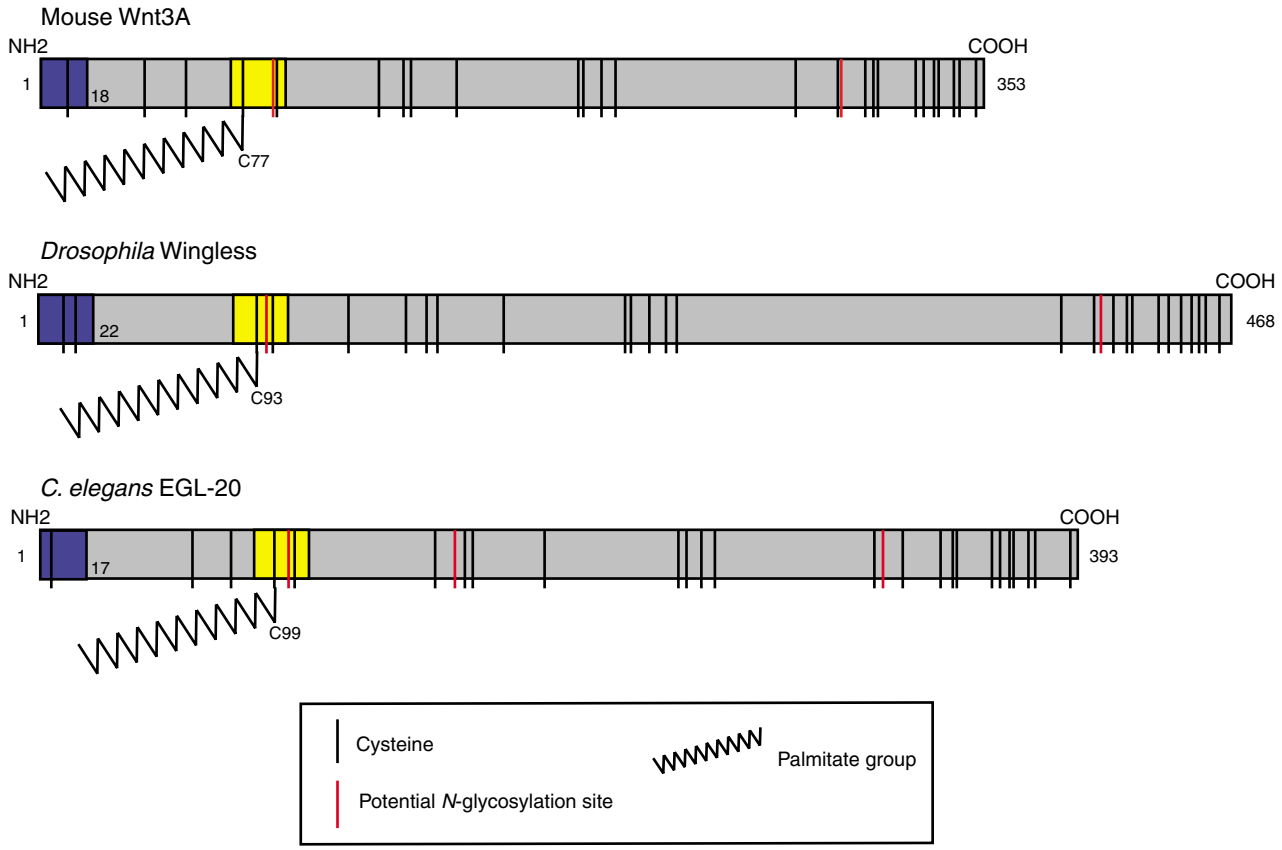

Fig. 1. A schematic of mouse Wnt3A, *Drosophila* Wingless and the *C. elegans* Wnt protein EGL-20. Wnts do not contain specific domains, but harbour an average of 23 to 25 cysteines that are highly conserved between different species. One of the *N*-terminally located cysteines is palmitoylated by the *O*-acyltransferase Porcupine, which binds Wnt in the corresponding region (yellow box). Wnts also contain a number of potential *N*-glycosylation sites, the functions of which are unknown. The signal sequence is represented in blue.

post-translational modifications. With respect to the *N*-glycosylation, in the absence of Porcupine, the attachment of the oligosaccharide chains is impaired. Conversely, Porcupine overexpression results in ectopic glycosylation of Wg (Tanaka et al., 2002). The precise role of Porcupine in this process is unknown. Interestingly, the Porcupine *C*-terminal region binds an *N*-terminal domain of Wg that contains conserved cysteines. Furthermore, Wg *N*-glycosylation competes with the formation of intracellular disulfide bonds, as suggested by the enhanced glycosylation observed after treatment by a reducing agent such as Dithiotreitol (DTT) (Tanaka et al., 2002). Porcupine may therefore protect these residues from forming disulfide bonds. This theory was initially supported by the absence of additional effects of Porcupine overexpression under fully reducing conditions. However, Porcupine does not affect the formation of disulfide bonds (Tanaka et al., 2002), although a transient effect delaying this process, which would promote Wg glycosylation, cannot be ruled out. Another model can be envisaged where Porcupine targets Wnt to the ER membrane, facilitating its modification by the OST complex. This may be directly achieved by the physical binding of Wnt to membrane-bound Porcupine, or indirectly by the Porcupine-dependent acylation of Wnt (see below). Consistent with this model, a membrane-tethered form of Wg is more efficiently glycosylated (Tanaka et al., 2002). In addition to targeting Wnt to the ER membrane, the binding of Porcupine may have an effect on Wnt conformation, allowing optimal access of the OST complex to the different *N*-glycosylation sites.

Despite the lack of direct evidence, the acyltransferase Porcupine has also been suggested to catalyze the palmitoylation of Wnt. First, Porcupine is required for Wg hydrophobicity and membrane association (Zhai et al., 2004). Second, the Porcupine-binding domain of Wg spans the region where the modified cysteine is located (Fig. 1) (Tanaka et al., 2002). Furthermore, similarly to loss of palmitoylation, loss of Porcupine abrogates Wg signalling (Riggleman et al., 1990; van den Heuvel et al., 1993). However, in *Drosophila* embryos, Wg secretion is inhibited in the absence of Porcupine, whereas Wnt3A(C77A), lacking the palmitoylated cysteine, is secreted normally in mammalian cell culture (Kadowaki et al., 1996; van den Heuvel et al., 1993; Willert et al., 2003), indicating that catalyzing the palmitoylation of this conserved cysteine is not the only function that Porcupine may play in the Wnt-maturation process. Recent studies may, nevertheless, confirm Porcupine as the Wnt-acylating enzyme. In addition to being palmitoylated at this *N*-terminally located cysteine, Burrus and colleagues found that Wnt is lipid-modified at other positions in a Porcupine-dependent manner as well. Indeed, Wnt3A(C77A) was shown to still harbour one or more lipid modifications despite lacking the palmitoylated cysteine. Additionally, overexpression of Porcupine further increased the fraction of Wnt3A(C77A) found in the detergent phase in phase separation experiments (L. Burrus, personal communication). This suggests that residues other than those previously described can be modified by Porcupine and can confer hydrophobicity. Interestingly, the group of Shinji Takada has shown that mutation of another specific amino acid in Wnt3A, besides C77,

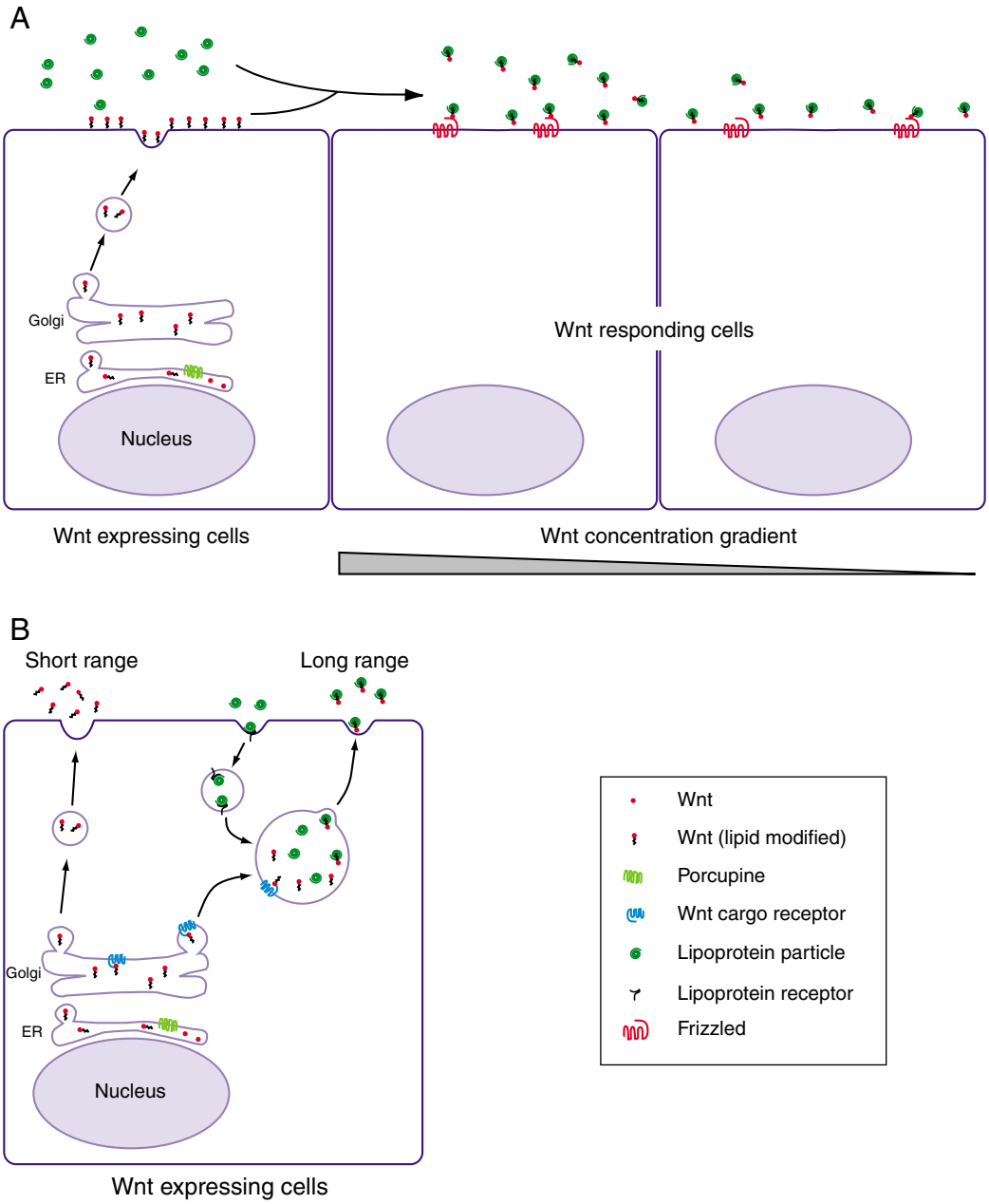

Fig. 2. Wnts associate with lipoprotein particles. Wnts associate with lipoprotein particles that may partially counteract the palmitate-mediated affinity of Wnt for cell membranes, thereby allowing the spreading of the protein further along the morphogenetic field. (**A**) This association could occur extracellularly. (**B**) Alternatively, as suggested by the potential sorting of Wnt to endosomes, the association of Wnt with lipoprotein particles may occur within the Wnt-producing cells, after endocytosis and the endosomal targeting of extracellular lipoprotein particles. Only a low fraction of Wnt binds lipoprotein particles, suggesting that long-range-acting Wnt may use a specialized secretion route, whereas short-range-acting Wnt may enter the constitutive secretion pathway.

prevents all lipid modification and results in the retention of the protein in the ER (S. Takada, personal communication). The different lipid groups may therefore have separate functions. The previously identified palmitoylation may affect Wnt function itself, either by allowing its sorting to specialized compartments where Wnt acquires crucial features, or by enhancing its signalling potential at the level of the responding cells. Additional acylation(s) may be required for the proper sorting of Wnt out of the ER and may therefore affect its secretion. All those modifications appear to be dependent on Porcupine, explaining the retention of Wnt within its producing cells when Porcupine function is inhibited.

Intracellular trafficking of Wnt

Before being released into the extracellular space, Wnts within the producing cells move from the ER to the trans-Golgi network (TGN), and from there through different subcellular compartments (Gonzalez et al., 1991). It is tempting to speculate that fully modified and functional Wnt can leave the ER and enter the bulk flow secretory pathway, thereby exiting its expressing cells via unregulated secretion vesicles. However, some results suggest that Wnt may use an alternative secretion route. First, in Wg-expressing cells, a fraction of Wg has been found to exist in the endosomal compartment (notably in multi-vesicular bodies), as well as in

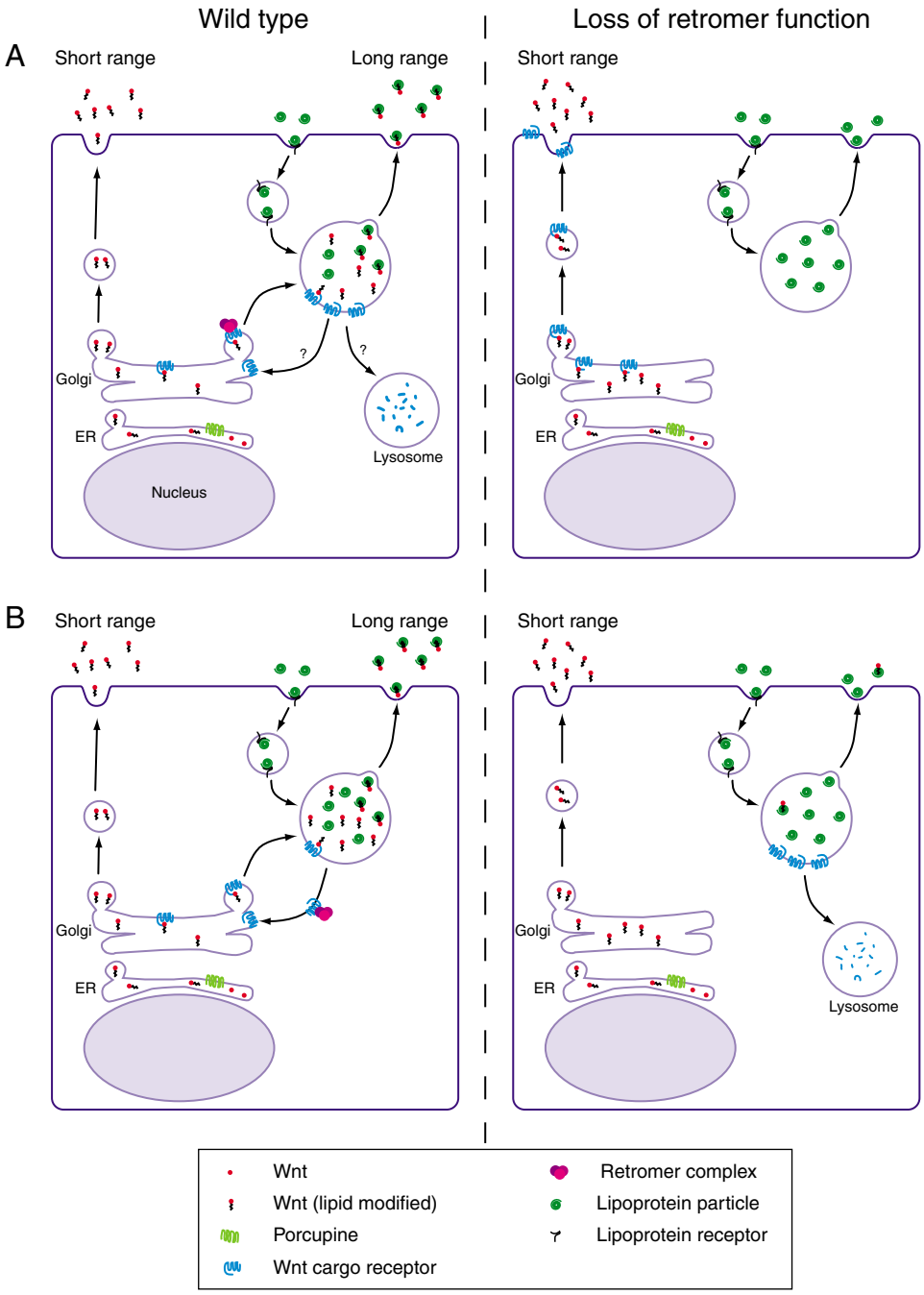

Fig. 3. Two models of the role of the retromer complex in Wnt maturation. (**A**) The retromer may directly or indirectly bind a putative Wnt cargo receptor in the TGN, directing the sorting of the Wnt–Wnt-cargo-receptor complex to the endosomes for association with lipoprotein particles (left panel). From the endosomes, the Wnt cargo receptors may either be recycled to the Golgi or degraded in the lysosomes. In the absence of retromer function, all Wnts enter the constitutive secretion pathway and exit the cells in their short-range-signalling form (right panel). (**B**) The retromer complex may retrieve the Wnt cargo receptors from the endosomes to the TGN, allowing the further cycling of Wnt molecules (left panel). In the absence of retromer function, the Wnt cargo receptors accumulate in the endosomes and are eventually degraded in the lysosomes. A shortage of Wnt cargo receptors in the TGN prevents the sorting of Wnts to the endosomes, resulting in constitutive secretion of short-range-acting Wnts (right panel).

recycling vesicles that potentially arise from endosomes and are directed to the plasma membrane (Gonzalez et al., 1991; Pfeiffer et al., 2002; van den Heuvel et al., 1989). This localization may result from the direct endocytosis of secreted Wg by the producing cells themselves. Alternatively, Wg may be actively targeted to this subcellular compartment within the expressing cells, before secretion. Second, the comparison of Wnt with Hedgehog also supports the hypothesis of a specialized secretion pathway. Wnt and Hedgehog are morphogens that share several properties at the structural (both are lipid-modified) and functional levels (Nusse, 2003). A recent study showed that Hedgehog does not enter the default secretory pathway (Gallet et al., 2003): by monitoring Hedgehog localization in its producing cells, the authors found that it segregates away from a secreted form of GFP. Wnt may follow a similar route. Finally, Wg has also been found to be associated with lipid rafts in cell culture (Zhai et al., 2004). Lipid rafts are separate microdomains of ordered lipid phases within the bilayer of the membrane and are known to be involved in multiple aspects of membrane transport (Ikonen, 2001). It is especially interesting to notice that rafts can act as platforms that segregate subgroups of proteins from other secreted proteins, partitioning them into specialized sorting and secreting routes (Schmidt et al., 2001).

Nothing is known about the specific recognition signals involved in the potential intracellular sorting of Wnt. *N*-glycosylation, as well as lipid modification, may play a role in this process. Indeed, palmitoylation of Wg is required for its association with lipid rafts (Zhai et al., 2004). Furthermore, a putative Wnt cargo receptor at the membrane of the TGN may bind Wnt and direct its transport to the endosomal compartment. Again, lipid rafts have been shown to mediate the inclusion of sorting receptors and cargo molecules into

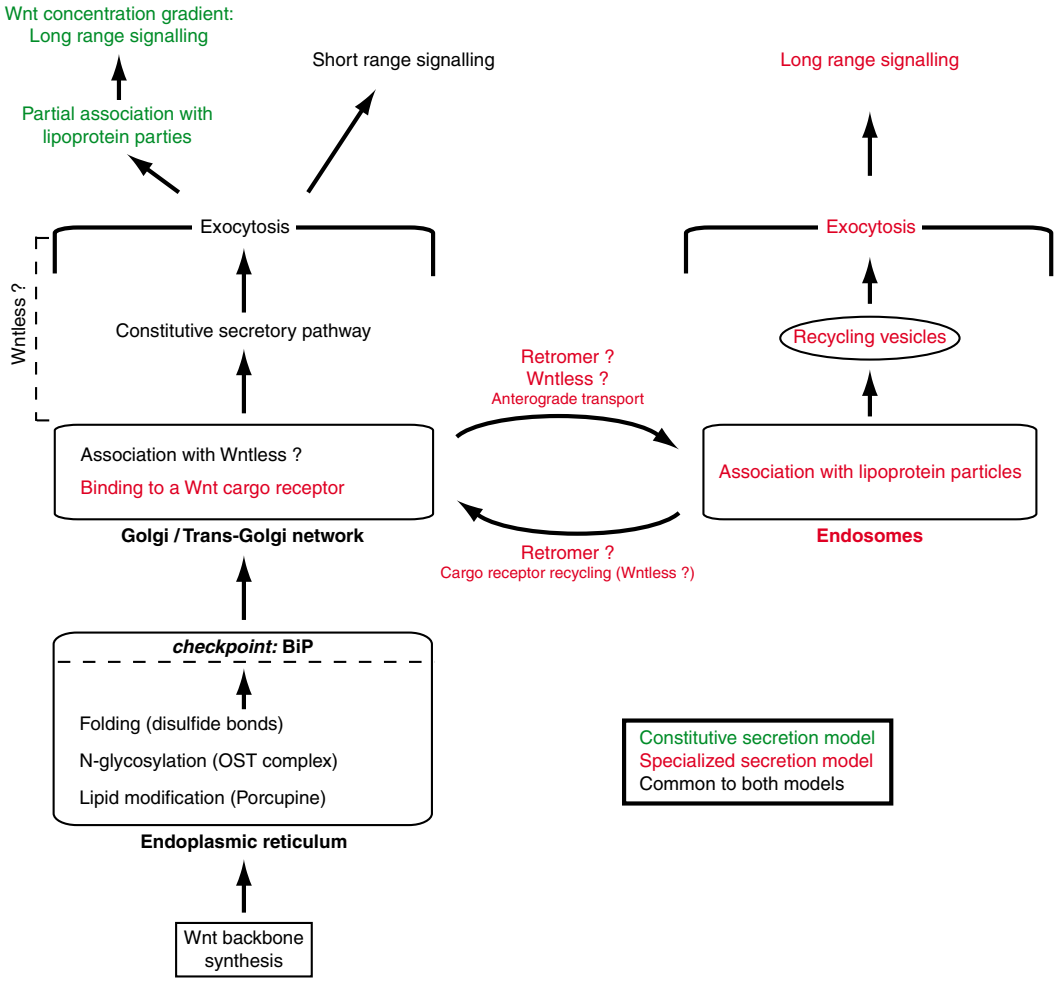

Fig. 4. Production of a fully functional Wnt by Wnt-expressing cells. A schematic of some of the different models proposed in this review. The steps specific to the model where Wnt is secreted via the constitutive secretory pathway and associates with lipoprotein particles outside of the cells are in green. The steps specific to the specialized sorting route for endosomal association with lipoprotein particles and secretion of long-range-signalling Wnt are in red. The steps common to the two models are in black.

the forming vesicle (Dhanvantari and Loh, 2000). The existence of such a cargo receptor has not yet been reported. However, the transmembrane protein Wntless/Evi, which is required within Wnt-producing cells, may be a good candidate for this mechanism (see below). Although a requirement for additional intracellular sorting events cannot be ruled out, Wnt is likely to leave the endosomal compartment via recycling vesicles, resulting in its release outside of its producing cells.

The sorting of Wnt to different subcellular compartments may play a key role in its maturation process, and a very precise description of Wnt subcellular localization in the producing cells will be necessary to reconstruct its exact secretion route. Indeed, efficient short- and long-range Wnt signalling may depend on more than one secretion route. Furthermore, a question arises about the necessity for Wnt to transit via the endosomal compartment. A recent study on the association of Wg and Hedgehog with lipoprotein particles, discussed below, may give some insight into this process.

Wnt is associated with lipoprotein particles

As has been thoroughly demonstrated for Wg in *Drosophila* (Neumann and Cohen, 1997; Strigini and Cohen, 2000; Zecca et al., 1996), in other species, Wnts also act as morphogens and can reach responding cells that are located several cell diameters away from the Wnt source (Coudreuse et al., 2006; Kiecker and Niehrs, 2001). The migration of Wnt along the morphogenetic field, and whether this occurs via extracellular diffusion or transcytosis, has long been a subject of debate (Vincent and Dubois, 2002). Although some discrepancies remain, the majority of recent studies favour a model in which Wnt can spread in a controlled, but extracellular, manner (Baeg et al., 2001; Han et al., 2005; Lander et al., 2002; Strigini and Cohen, 2000).

Wg was recently found to associate with lipoprotein particles (Panakova et al., 2005). Lipoprotein particles consist of phospholipid monolayers that surround a core of esterified cholesterol and triglycerides, scaffolded by members of the apolipoprotein family. In *Drosophila*, the formation of such a Wg–lipoprotein-particle complex is required for long-range signalling in the wing imaginal disc, but it does not seem to dramatically influence short-range effects of Wg. The function of this association in long-range signalling of Wnt is unclear, and it has so far not been formally proven that this complex has signalling activity. Several models can, however, be envisaged. First, lipoprotein particles may displace hydrophobic Wnt from the membranes of its producing cells and from cells located in their direct vicinity, thereby allowing its further spreading. Second,

it may tightly regulate the extension and steepness of the Wnt gradient by mediating Wnt interactions with members of the heparan sulfate proteoglycan family (HSPG) (Baeg et al., 2001; Giraldez et al., 2002; Han et al., 2005; Kirkpatrick et al., 2004; Kreuger et al., 2004). Third, although possibly counteracting its membrane targeting at the site of production, the transport of Wnt on lipoprotein particles may prevent its dilution in the extracellular space and therefore increase its local concentration at its receptors. This may be achieved directly or indirectly by interaction with HSPGs. Finally, lipoprotein particles may stabilize extracellular Wnt and allow the clustering of Wnt molecules, facilitating the presentation of efficient amounts of Wnt to the receptors. This may be especially important for signalling at a distance from the source, where the concentration of Wnt becomes limiting.

The manner and location in which Wnt may associate with these lipoprotein particles is particularly interesting in the context of this review. Consistent with the displacement hypothesis, one model suggests that Wnt binds lipoprotein particles outside of its producing cells, after secretion (Fig. 2A). However, the potential sorting of Wnt to endosomes suggests an alternative process. Thus, lipoprotein particles may be endocytosed by low density lipoprotein (LDL)- or high density lipoprotein (HDL)-receptors at the surface of the Wnt-producing cells. Internalized lipoprotein particles may then reach the endosomes where the mature Wnt has been sorted to. The association of Wnt with these particles may therefore occur within the Wnt-producing cells, before Wnt secretion (Fig. 2B). The requirement for a mechanism that extracts Wnt out of the default secretory pathway may therefore be related to the binding of Wnt to lipoprotein particles. However, Wnt is likely to use both the constitutive and a specialized secretion route. Indeed, only a low fraction of total Wg was found to be bound to lipoprotein particles (Panakova et al., 2005). Most Wnt may therefore use the default secretory pathway and signal at a short distance from the producing cells, whereas a small fraction of Wnt, dedicated to long-range signalling, may be sorted to the endosomes to be associated with lipoprotein particles (Fig. 2B). Endosomal budding and formation of recycling vesicles may subsequently release the Wnt–lipoprotein-particle complex outside of the producing cells, allowing optimal signalling along the morphogenetic field.

Wntless/Evi and Wnt secretion

Irrespective of the pathway, Wnt secretion appears to be tightly regulated by the highly conserved seven-pass transmembrane protein Wntless/Evi (Wls), which physically interacts with Wnt (Banziger et al., 2006; Bartscherer et al., 2006). In the absence of Wls, Wnt is retained within its producing cells, resulting in a Wnt loss-of-function phenotype. The function of Wls in Wnt signalling is conserved in *C. elegans*, where the Wls orthologue MOM-3 (also known as MIG-14) is required for signalling by all Wnts tested (Eisenmann and Kim, 2000; Harris et al., 1996; Thorpe et al., 1997). Previous results from genetic studies on MIG-14, and especially the recent analysis of Wls function in *Drosophila* and mammalian cells (Banziger et al., 2006; Bartscherer et al., 2006), have demonstrated that it is specifically required within the Wnt-producing cells for Wnt secretion.

Conflicting results on Wls subcellular localization have been obtained by different groups. The Boutros laboratory identified Wls mainly at the plasma membrane of *Drosophila* imaginal disc cells, whereas Bänziger and colleagues suggest that Wls is located in the Golgi, and in vesicles between the Golgi and the cell surface in *Drosophila*, as well as in mammalian cells (Banziger et al., 2006; Bartscherer et al., 2006). However, such differences may result from variations in the expression levels of the Wls transgenes. The potential localization of Wls in vesicles suggests that it may regulate the intracellular trafficking of Wnt between different compartments of the producing cells. Mis-sorting of Wnt to certain compartments may preclude its secretion. Consistent with this hypothesis, in the absence of Wls, Wnt3A does not reach the surface of its producing cells. In the study by the Basler laboratory, the subcellular localization of Wnt3A was not noticeably modified by the loss of Wls function in cell culture (Banziger et al., 2006). However, it cannot be ruled out that the sorting defect is relatively subtle and only detectable by a detailed compartmental analysis, or by co-fractionation experiments. Interestingly, Bartscherer and colleagues show that the apical localization of Wg in the wing disc epithelium is lost in the absence of Wls (Bartscherer et al., 2006). Instead, Wg appears to be located throughout the expressing cells. Thus, in a Wls-mutant background, Wnt may not reach the appropriate apical compartment or the compartment where it acquires its capacity to be apically sorted and secreted. However, the interpretation of this defect in apical localization remains difficult, as Wls was shown to be required for Wnt secretion in non-polarized assays (Banziger et al., 2006).

The aberrant sorting of Wnt during its maturation may also indirectly disrupt its secretion. Similarly to the loss of Porcupine or mutation of a specific residue in Wnt3A (S. Takada, personal communication), the mis-sorting of Wnt may prevent post-translational modifications that are essential for its secretion. However, Porcupine-mediated acylation itself is unlikely to be affected by Wls, because Wls was not detected in the ER and was found to act later in the secretion process. Furthermore, inhibition of Wnt3A acylation results in its retention in the ER, whereas Wg is localized throughout the expressing cells in the absence of Wls function. Finally, *N*-glycosylation of Wnt3A, which may serve as a signal for its polarized secretion, was found to be intact in the absence of Wls (Banziger et al., 2006). Wls-dependent trafficking of Wnt may, therefore, regulate other, as yet unknown, modifications. Alternatively, Wls may allow the optimal post-translational modification of Wnt by means other than the control of its subcellular localization. Thus, Wls may directly act as a chaperone and notably control the modification of residues that are crucial for Wnt secretion. This might be achieved by presenting Wnt to the appropriate enzyme or by facilitating the access of the enzyme to the correct residues.

Our currently limited understanding of Wnt maturation allows only for speculative models, and it cannot be ruled out that Wls controls unknown steps of Wnt production that are required for its secretion. A detailed description of the biochemical properties of mature Wnt, as well as a complete study of the route followed by Wnt during its production by the Wnt-expressing cells, will be necessary to understand the functions of proteins such as Wls.

The role of the retromer complex

An additional level of complexity has recently been added to this picture with the identification of the retromer complex as a key player in the production of a functional Wnt (Coudreuse et al., 2006). The retromer is a highly conserved multiprotein complex, the core of which consists of the subunits Vps35, Vps29 and Vps26 (Seaman et al., 1998). It was initially discovered in yeast for its role in the intracellular sorting of the carboxypeptidase Y (CPY) cargo receptor Vps10p (Paravicini et al., 1992; Seaman et al., 1997). Vps10p binds CPY (which are lysosomal enzymes) in the TGN and ensures their transit to late endosomes. After the pH-dependent release of CPY in endosomes, Vps10p is recycled back to the TGN.

This retrograde transport is mediated by the interaction of the cytoplasmic tail of Vps10p with Vps35p (Nothwehr et al., 2000). In the absence of retromer function, Vps10p accumulates in the endosomes and is eventually targeted to the lysosomes. Vps10p subsequently becomes limiting in the TGN, resulting in the secretion of CPY via the constitutive secretion pathway. Similarly, the mammalian mannose 6-phosphate receptor, which allows the transport of lysosomal hydrolase precursors, is recycled to the TGN by the retromer complex (Arighi et al., 2004). In mammalian cells, the retromer complex was also shown to mediate basal-to-apical transcytosis of the immunoglobulin-A–immunoglobulin-receptor complex (Verges et al., 2004).

A recent study using both *C. elegans* and vertebrate systems suggests that the retromer is also required within the Wnt-producing cells for proper Wnt signalling (Coudreuse et al., 2006). Thus, loss of the core protein VPS-35 abrogates EGL-20/Wnt signalling in *C. elegans* (see Prasad and Clark, 2006). Similarly, the knockdown of Vps35 in mammalian cells and *Xenopus tropicalis* inhibits Wnt target gene expression (Coudreuse et al., 2006). In *C. elegans*, retromer function appears to be specific to Wnt signalling, as no other major signalling pathway is significantly affected in mutants of retromer components. Interestingly, the retromer was found to be required within the EGL-20/Wnt-producing cells for the formation of an anteroposterior gradient of EGL-20. Consistent with these findings, loss of the retromer was shown to fully impair long-range, but to only mildly affect short-range, EGL-20 signalling. Finally, phenotypic studies in *C. elegans* and biochemical analyses in mammalian cells have shown that Wnt secretion itself is not inhibited in the absence of retromer function (Coudreuse et al., 2006).

Several models can be proposed for the function of the retromer in Wnt-gradient formation. As discussed earlier, Wnt harbours post-translational modifications, such as *N*-glycosylations and palmitoylations. Additional, as yet unidentified, modifications may also occur during Wnt maturation. Some of these modifications, acquired in various specialized subcellular compartments of the expressing cells, may be required for the formation of a Wnt gradient, and therefore for the long-range signalling activity of Wnt. Thus, the retromer may promote Wnt maturation by keeping the different Wnt-modifying enzymes in their assigned compartments. This may be achieved through the direct binding of the retromer complex to the cytoplasmic tail of membrane-bound enzymes, or by the interaction of this complex with associated cargo receptors. As a member of the MBOAT family, Porcupine localization may be directly or indirectly regulated by the retromer complex. However, Porcupine is required for Wg secretion, whereas loss of retromer function does not affect Wnt secretion, indicating that the retromer is probably not required for Porcupine function.

Interestingly, the effect of loss of retromer function is strikingly similar to the effect observed for Wg and Hedgehog when their association with lipoprotein particles is impaired (Panakova et al., 2005). Thus, in the absence of the *Drosophila* lipoprotein lipophorin, which is an essential component of the lipoprotein particles in *Drosophila*, long-range Wg signalling is more strongly affected than is short-range Wg signalling. If the binding of Wnt to lipoprotein particles occurs within the endosomal compartment after sorting of Wnt by a cargo receptor, at least two functions can be envisaged for the retromer complex. First, the retromer may directly bind the cytoplasmic tail of the Wnt cargo receptor at the TGN and allow the continuous transport of a fraction of newly synthesized Wnt to the endosomes (Fig. 3A). Alternatively, it may promote the binding of a cytoplasmic transporter to the Wnt cargo receptor. In the absence of retromer function, the Wnt–Wnt-cargo-receptor complex may accumulate in the TGN. Wnt may subsequently be released and enter the bulk flow secretion. This dissociation may occur within the TGN, or at later stages, because of a change in chemical environment along the default secretory pathway. Consequently, the endosomal association of Wnt with lipoprotein particles is prevented, resulting in constitutive secretion of a non-spreading, short-range-signalling form of Wnt. A second model emerges from the known recycling function of the retromer in yeast. This model implies that the amount of Wnt cargo receptor at the TGN is limiting. Thus, similar to its function in Vps10p recycling, the retromer may retrieve Wnt cargo receptors from the endosomes to the TGN, allowing additional rounds of Wnt cycling (Fig. 3B). In the absence of retromer function, the Wnt cargo receptors may accumulate in the endosomes and may eventually be degraded in the lysosomes. The subsequent shortage in cargo receptors would prevent Wnt from exiting the TGN. Again, this would result in constitutive secretion of a short-range signalling form of Wnt. In addition to requiring retromer function, Wnt association with lipoprotein particles may also depend on the *N*-terminal palmitate group. Thus, when the palmitoylated cysteine is mutated into a serine, EGL-20 is similarly unable to form an anteroposterior gradient in *C. elegans* (our unpublished data).

These two models, however, rely on the actual sorting of Wnt to the endosomes before secretion as well as on the hypothetical existence of a Wnt cargo receptor. Furthermore, they imply that the association of Wnt with lipoprotein particles, which is necessary for the spreading of Wnt along the morphogenetic field, is not the means by which hydrophobic Wnt avoids sequestration at the membrane of cells directly adjacent to its producing cells. Indeed, Wnt3A was found in normal amounts in the medium of vertebrate cells after the depletion of Vps35 by RNAi, suggesting that it does not stick to the nearby membranes despite the potential absence of lipoprotein-particle association (Coudreuse et al., 2006). Further studies are obviously required to understand the exact role of the lipoprotein particles and the consequences of the loss of retromer function on the association of these particles with Wnt.

Wls, the retromer complex and the association of Wnt with lipoprotein particles

Both Wls and the retromer complex may be involved in intracellular trafficking of Wnt within Wnt-expressing cells. It is therefore attractive to speculate on models in which Wls and the retromer functionally interact to allow the efficient maturation of Wnt and its association with lipoprotein particles. The molecular nature of Wls, a transmembrane protein that binds Wnt, suggests that it may act as the previously discussed Wnt cargo receptor, allowing the transport of Wnt from the TGN to the endosomes. In this context, the retromer may directly or indirectly bind Wls and mediate the anterograde transport of the Wls-Wnt complex from the TGN. Alternatively, it may regulate the retrieval of Wls from the endosomes, thereby ensuring the further cycling of Wnt molecules. However, such models seem inconsistent with the inhibition of Wnt secretion observed in the absence of Wls, whereas normal amounts of Wnt appear to exit the cells when retromer function is impaired. Further investigation is therefore needed to reconcile these results and to demonstrate if Wls, the retromer and the association of Wnt with lipoprotein particles are different steps of the same mechanism (Fig. 4).

Conclusions

The question of the maturation, sorting and secretion of a functional, long-range-acting Wnt protein has long been left aside in favour of the study of Wnt signal transduction downstream of its receptors.

However, it has recently become clear that the function of Wnt in vivo and its capacity to spread and precisely pattern the developing embryo are dependent on key processes that occur within Wnt-producing cells before secretion. Specific post-translational modifications, the controlled sorting of Wnt to specialized subcellular compartments and its association with extracellular vehicles could be at the tip of the iceberg of mechanisms that contribute to the release of an optimal Wnt protein. A concerted effort to investigate these processes needs to be made in order to propose new models and to elucidate some of the many questions and contradictions that we have highlighted in this review. The better characterization of the properties that Wnt acquires during its maturation, as well as an in-depth description of Wnt biochemistry, are inevitable steps towards a better understanding of its function in animal development.

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