1 Wnt Signal Production, Secretion, and Diffusion

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Introduction

Wnt proteins are members of an evolutionarily conserved family of secreted signaling proteins that play a central role in the development of metazoan organisms (Willert and Nusse, 2012) (see Chapter 12). Wnts are lipid-modified glycoproteins that can signal in a short-range manner to target cells that are directly adjacent to Wnt-producing cells (Sato *et al*., 2011). Importantly, Wnts can also form long-range concentration gradients that provide positional information to cells in developing tissues (Zecca, Basler, and Struhl, 1996). The formation and regulation of such morphogenic gradients is one of the major enigmas in the Wnt field, raising questions on how the hydrophobic Wnt protein is efficiently released from producing cells and on how it spreads in the aqueous extracellular environment of the tissue. In this chapter, we will briefly discuss the lipid and sugar modification of Wnt proteins and then focus on the specialized secretion machinery that mediates the release of Wnt from producing cells and the mechanisms that facilitate and control the spreading of Wnt in morphogen gradient formation.

Posttranslational modification of Wnt

Biosynthesis of Wnt proteins is initiated by their translation and translocation in the rough endoplasmic reticulum (ER), after which the Wnt proteins undergo a number of maturation and modification steps (Figure 1.1, step 1). First, all Wnt proteins harbor a large number of conserved cysteines (23–25 on average), which participate in the formation of intramolecular disulfide bonds (Janda *et al*., 2012). In addition, Wnts undergo two major types of posttranslational modification, N-glycosylation and lipidation. Although the addition of *N*-glycans may facilitate the secretion of a subset of Wnts, they appear generally dispensable for the activity of mature Wnt proteins, as glycosylation-deficient mutants exhibit no major defects in signaling (Doubravska *et al*., 2011; Komekado *et al*., 2007; Kurayoshi *et al*., 2007; Tang *et al*., 2012). In agreement, the two glycan groups attached to *Xenopus* Wnt8 (XWnt8) did not contribute to the Wnt–Frizzled (Fz) interaction in the recently solved crystal structure (Janda *et al*., 2012).

A number of studies have reported on the modification of both vertebrate and invertebrate Wnts with two acyl groups: a palmitate at an

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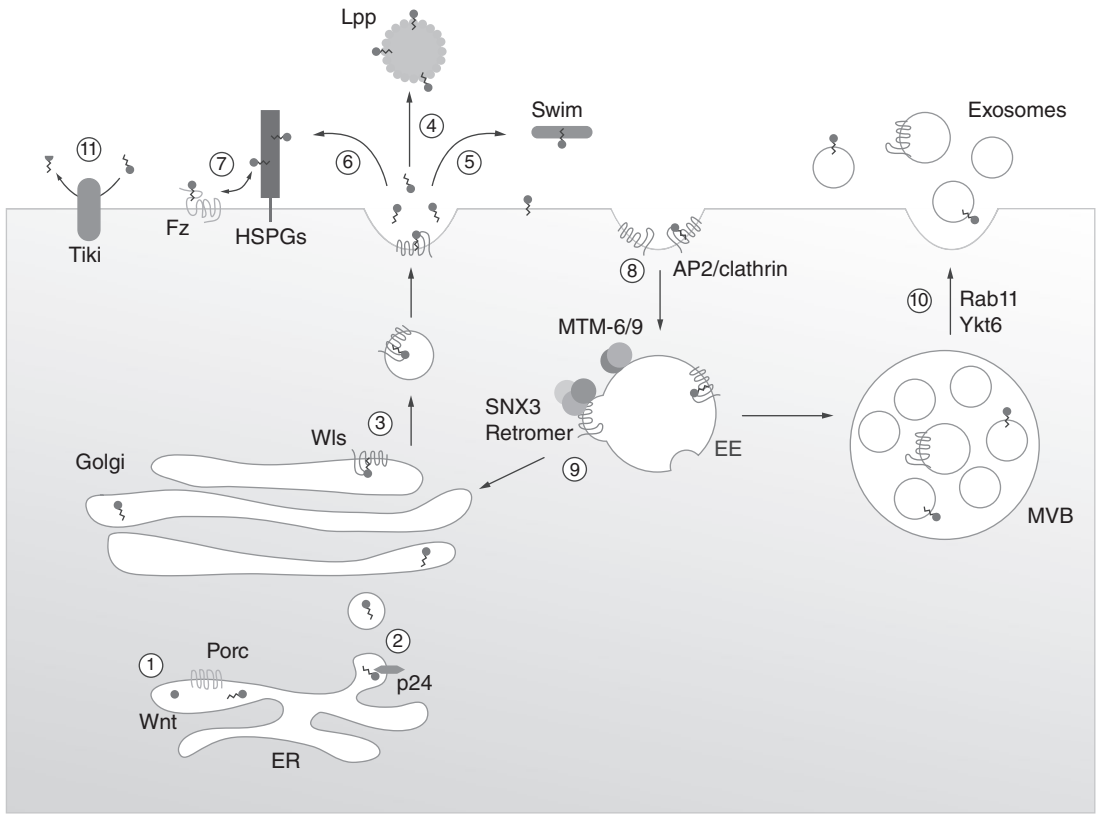

Figure 1.1 Wnt production, secretion, and spreading mechanisms. Wnt is lipid modified by Porc in the ER (1) and is transported to the Golgi through a p24-dependent mechanism (2). Next, Wnt binds the sorting receptor Wls, which transports Wnt to the cell surface (3). Release and diffusion of Wnt is facilitated by binding to lipoprotein particles (4), the lipocalin Swim (5), or HSPGs such as Dally and Dlp (6). HSPGs may also function as coreceptors that promote binding of Wnt to the Fz receptor (7). Wls is recycled from the plasma membrane through AP2-/clathrin-mediated endocytosis (8) and retromer-dependent endosome to Golgi retrieval (9). Wls and Wnt can also be internalized on intraluminal vesicles and be secreted on exosomes (10). The activity of secreted Wnt is modulated by Tiki (11). ER, endoplasmic reticulum; EE, early endosome; MVB, multivesicular body.

N-terminal cysteine and a palmitoleic acid at an internal serine, exemplified by the Cys93 and Ser239 residues, respectively, in the *Drosophila* Wnt family member Wingless (Wg) (Doubravska *et al*., 2011; Galli *et al*., 2007; Kurayoshi *et al*., 2007; Miura and Treisman, 2006; Takada *et al*., 2006; Willert *et al*., 2003). Genetic evidence strongly suggests that the ER-resident multispan *O*-acyltransferase Porcupine (Porc) is responsible for the acylation of Wnts (Zhai, Chaturvedi, and Cumberledge, 2004). *Porc* mutants show ER accumulation of Wnts, disrupted secretion, and reduced hydrophobicity of Wnt proteins (Zhai, Chaturvedi, and Cumberledge, 2004). Thus, Porc is required for the lipidation of Wnt proteins and subsequently drives ER exit and entry of Wnts into the secretory pathway. To understand how Wnt acylation impacts on protein function, Wnt mutants that lack the cysteine and serine acyl attachment sites were used in both cell culture and developmental studies (Tang *et al*., 2012). While palmitoleic acid modification at Ser239 was found essential for Wnt secretion and signaling (Tang *et al*., 2012), the palmitate at Cys93 appeared of less importance in the regulation of Wnt signaling *in vivo* (Tang *et al*., 2012). Intriguingly, the analogous *N*-terminal cysteine (Cys55) in the XWnt8 crystal structure is engaged in the formation of a disulfide bond that is predicted to be conserved across all Wnts (Janda *et al*., 2012). Thus, phenotypes observed

for Cys-to-Ala mutants may have resulted from conformational alterations in the protein, due to the absence of this important disulfide bond. The essential signaling role of the acyl modification of Ser was confirmed by the structure as this lipid moiety directly engaged a groove on the extracellular cysteine-rich domain (CRD) of Fz8 (Janda *et al*., 2012).

The Wnt secretion pathway

Once the Wnt protein is lipid modified and glycosylated, it is transported to the cell surface for release. Current evidence suggests that this is mediated through a specialized trafficking pathway (Lorenowicz and Korswagen, 2009; Port and Basler, 2010) and that different release mechanisms may contribute to the formation of distinct pools of Wnt that have different signaling activities in the tissue (Beckett *et al*., 2013; Gross *et al*., 2012; Panakova *et al*., 2005).

p24 proteins mediate ER to Golgi transport of Wnt

The first leg in the journey of Wnt to the cell surface is transport from the ER to the Golgi network (Figure 1.1, step 2). It has recently been shown that members of the p24 cargo adaptor family play a central role in this trafficking step (Buechling *et al*., 2011; Port, Hausmann, and Basler, 2011). Using large-scale RNA interference (RNAi) screens, it was found that the knockdown of the p24 family members Éclair, Emp24, and Opossum leads to a reduction in Wg secretion from Schneider 2 (S2) cells. Further experiments showed that these p24 family members are also required for Wg secretion *in vivo*. Thus, the knockdown of Éclair, Emp24, and Opossum in the wing imaginal disc resulted in the accumulation of Wg in producing cells, a reduction in target gene expression, and defects in the formation of wing margin tissue, a hallmark of defective Wg signaling. Importantly, Éclair, Emp24, and Opossum were not required for general protein secretion, as the ER export of the heparan sulfate proteoglycans (HSPGs) Dally and Dally-like (Dlp) and the Wnt secretion factor Wntless (Wls) (see succeeding text) were not affected.

p24 proteins have been proposed to function as cargo-specific adaptors that facilitate the sorting of cargo proteins into COPII vesicles, a class of transport carriers that mediate the trafficking of cargo proteins from the ER to the Golgi (Castillon *et al*., 2011). Emp24 and Opossum interact with Wg in coimmunoprecipitation experiments, indicating that they may have a similar cargo adaptor function towards Wnt proteins. In support of such a role is the observation that Wg accumulates in the ER in the absence of Éclair and Emp24 (Port, Hausmann, and Basler, 2011). Interestingly, the secretion of the non-lipid-modified *Drosophila* Wnt protein WntD is dependent on Opossum as well (Buechling *et al*., 2011). The role of Opossum in WntD secretion may, however, be indirect, as WntD does not bind to Opossum in coimmunoprecipitation experiments.

The Wnt binding protein Wntless is essential for Wnt secretion

The second stage in the transport of Wnt to the cell surface is mediated by Wls (Banziger *et al*., 2006; Bartscherer *et al*., 2006; Goodman *et al*., 2006), a protein that is also known as Evi or Sprinter in *Drosophila*, MIG-14 in *Caenorhabditis elegans* (Banziger *et al*., 2006), and GPR177 in the mouse (Fu *et al*., 2009). Like Porc, Wls is essential for Wnt secretion. In the *Drosophila* wing imaginal disc, for example, the loss of *wls* leads to the accumulation of Wg in producing cells and a strong reduction in Wg signaling (Banziger *et al*., 2006; Bartscherer *et al*., 2006), and also in the mouse and in *C. elegans*, mutation of *Gpr177* and *mig-14* disrupts Wnt signaling (Fu *et al*., 2009; Harris *et al*., 1996; Thorpe *et al*., 1997; Yang *et al*., 2008). This function appears to be specific to Wnt, as the loss of Wls does not affect general protein secretion or the release of the related lipid-modified morphogen Hedgehog (Hh) (Banziger *et al*., 2006). Wls encodes a highly conserved seven-pass transmembrane protein that binds Wnt in coimmunoprecipitation experiments (Banziger *et al*., 2006; Bartscherer *et al*., 2006). This interaction requires Porc and the conserved lipid-modified internal serine residue in Wnt proteins (Ser239 in Wg and Ser209 in Wnt3a), indicating that the addition of a palmitoleic acid chain at

this position is essential for binding to Wls (Coombs *et al*., 2010; Herr and Basler, 2012). This is consistent with the observation that the non-lipid-modified Wnt protein WntD does not bind to Wls and also does not require Wls for its secretion (Ching, Hang, and Nusse, 2008; Herr and Basler, 2012). The region of Wls that is necessary for Wnt binding is contained within the first extracellular loop (Fu *et al*., 2009). Interestingly, this region is predicted to share structural similarities with proteins of the lipocalin family, which bind lipid moieties of proteins to enable their extracellular transport. An analogous interaction mechanism thus may be involved in the Wls–Wnt interaction (Coombs *et al*., 2010). What is the function of Wls in the Wnt secretion pathway? Endogenous as well as fluorescently tagged versions of Wls show a prominent localization to the Golgi network, the endosomes, and the plasma membrane (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008; Port *et al*., 2008; Yang *et al*., 2008). Taken together with the observation that Wg accumulates in the Golgi of *wls* mutant cells (Port *et al*., 2008), these findings led to the hypothesis that Wls functions as a sorting receptor that facilitates trafficking of Wnt from the Golgi to the cell surface for release (Lorenowicz and Korswagen, 2009; Port and Basler, 2010).

Wntless is recycled to maintain efficient Wnt secretion

Once Wls reaches the plasma membrane, it is internalized and retrieved back to the Golgi to take part in further rounds of Wnt secretion (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008; Pan *et al*., 2008; Port *et al*., 2008; Yang *et al*., 2008). Mutations that interfere with this recycling induce strong defects in Wnt signaling, indicating that Wls is a limiting component in the pathway that needs to be recycled to maintain efficient Wnt secretion. In *Drosophila*, the expression of Wls is independent of Wnt signaling (Herr and Basler, 2012). Interestingly, the mouse Wls ortholog *Gpr177* is a direct Wnt target gene, indicating that mammalian Wnt proteins may stimulate their own secretion by upregulating Wls expression (Fu *et al*., 2009). However, also in mammalian cells, interfering with Wls retrieval induces defects in Wnt secretion (Belenkaya *et al*., 2008), indicating that despite this potential positive feedback, Wls recycling is still necessary for efficient Wnt secretion.

The first step in the recycling of Wls is internalization from the plasma membrane, which is mediated through AP2 adaptin- and clathrin-dependent endocytosis (Figure 1.1, step 8) and requires a conserved YXXΦ sorting motif that is present in the third intracellular loop of Wls (Gasnereau *et al*., 2011; Pan *et al*., 2008; Yang *et al*., 2008).

Next, Wls is retrieved from the endosomal system and is transported back to the Golgi (Figure 1.1, step 9) through a retromer-dependent trafficking pathway (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008; Port *et al*., 2008; Yang *et al*., 2008). The retromer is a multisubunit membrane coat complex that mediates the retrograde transport of cargo proteins such as the cation-independent mannose 6-phosphate receptor (CI-MPR) from endosomes to the *trans*-Golgi network (TGN) (Cullen and Korswagen, 2012; Seaman, 2005). The retromer consists of a stable trimer of the subunits Vps26, Vps29, and Vps35 that binds to a loosely defined sorting signal in the cargo protein and a membrane-bound heterodimer of the SNX–BAR sorting nexins SNX1/2 and SNX5/6. The SNX–BAR sorting nexins contain a membrane curvature-sensing BAR domain that drives the formation of membrane tubules into which cargo proteins such as the CI-MPR are sorted. Scission of these tubules generates transport carriers that deliver the cargo back to the TGN.

A role of the retromer in Wnt signaling was first discovered in *C. elegans*, where mutations in the cargo-selective subunits were found to induce a range of Wnt-related phenotypes (Coudreuse *et al*., 2006; Prasad and Clark, 2006). Subsequent studies showed that this function is evolutionarily conserved and that the retromer is required in Wnt-producing cells for Wls retrieval. The Vps26, Vps29, and Vps35 trimer binds to Wls in coimmunoprecipitation experiments (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008), and in the absence of retromer function, Wls fails to be retrieved from the endosomal system and is degraded in lysosomes (Belenkaya *et al*., 2008; Franch-Marro *et al*., 2008; Port *et al*., 2008; Yang *et al*., 2008). Interestingly, it was found that the endosome to TGN transport of Wls is independent of the

SNX–BAR sorting nexins (Harterink *et al*., 2011). Instead, Wls retrieval requires the unrelated sorting nexin SNX3, which sorts Wls into vesicular transport carriers that are morphologically distinct from the tubular carriers formed by the SNX–BAR sorting nexins. SNX3 is recruited to endosomal membranes through a phosphatidylinositol 3-monophosphate (PI3P)-binding PX domain. In *C. elegans*, this endosomal association is regulated by the myotubularin lipid phosphatases MTM-6 and MTM-9, and MIG-14/Wls retrieval is strongly disrupted in their absence (Silhankova *et al*., 2010). Why Wls retrieval is mediated through a specialized retromer pathway remains to be established.

Release of Wnt from producing cells

The first step in the release of Wnt is dissociation from Wls. This was shown to be dependent on vacuolar acidification, most likely of the secretory vesicles that transport the Wnt–Wls complex to the cell surface (Coombs *et al*., 2010). When vacuolar acidification is blocked, Wnt3a still reaches the cell surface, but is not released from Wls into the medium. Interestingly, a decrease in pH is not sufficient for the dissociation of Wnt from Wls, indicating that additional mechanisms are required for Wnt release. Such mechanisms may involve binding of Wnt to HSPGs on the surface of Wnt-producing cells or may require the presence of specific carriers.

A role for carriers in Wnt secretion and diffusion was first proposed based on the observation that Wg colocalizes with punctate structures in the *Drosophila* wing imaginal disc (Greco, Hannus, and Eaton, 2001). These so-called argosomes, which derive from the Wg-producing cells, were proposed to act as vehicles for Wg diffusion (Figure 1.1, step 4). Although the exact nature of these argosomes remains unknown, subsequent studies have provided evidence that they may represent lipoprotein particles or exosomes.

Density gradient centrifugation experiments with *Drosophila* larval extracts showed that Wg and Hh cofractionate with lipophorin (Panakova *et al*., 2005). Lipophorin is a component of lipoprotein particles, structures that consist of apolipoproteins and a phospholipid monolayer that surround a core of esterified cholesterol and triglycerides. Lipoprotein particles act as lipid carriers and would therefore be ideally suited to facilitate diffusion of Wg and Hh, which may bind to the particles through insertion of their fatty acid and cholesterol tails into the lipid core. Consistent with such a role, it was found that Wg colocalizes with lipophorin in the wing disc and that the knockdown of lipophorin interferes with Wg gradient formation, resulting in reduced expression of the long-range target gene *distalless (dll)* (Panakova *et al*., 2005). In addition to promoting diffusion, lipoprotein particles may also have a role in the release of Wnt from mouse fibroblast L cells, a cell line commonly used for mammalian Wnt secretion (Willert *et al*., 2003; Neumann *et al*., 2009). Thus, the secretion of Wnt3a from L cells into the medium requires the presence of low-density lipoprotein (LDL) and especially high-density lipoprotein (HDL) particles (Neumann *et al*., 2009). Interestingly, it was found that the release of Wnt3a from LDL receptor mutant Chinese hamster ovary (CHO) cells was strongly stimulated by the expression of the HDL receptor SR-BI/II, indicating that the SR-BI/II receptor may stimulate Wnt secretion by binding and releasing HDL particles. However, this does not appear to be a general mechanism, as the knockdown of SR-BI/II did not interfere with Wnt secretion from L cells (Neumann *et al*., 2009).

A study on Wg signaling in the neuromuscular junction of *Drosophila* revealed that Wls and Wg are present on small vesicles that traverse the synaptic cleft (Korkut *et al*., 2009). Interestingly, it was found that these vesicles are also formed by S2 cells and can be transferred between cells in tissue culture. Recently, three separate studies have shown that these vesicles are exosomes (Figure 1.1, step 10) (Beckett *et al*., 2013; Gross *et al*., 2012; Koles *et al*., 2012), but their function in Wnt release and signaling remains unclear.

Exosomes are small vesicles that are secreted from cells when multivesicular bodies fuse with the plasma membrane and release their content of intraluminal vesicles into the extracellular space (Simons and Raposo, 2009). Both Wls and Wg can be purified together with exosomes from the culture medium of Wg-expressing S2 cells (Beckett *et al*., 2013; Gross *et al*., 2012; Koles *et al*., 2012). This exosome fraction of Wg is

active but represents only part of the total amount of Wg present in the medium, indicating that secretion on exosomes acts in parallel to other release mechanisms. What is the function of this exosome-associated pool of Wnt? On this topic, disagreement is apparent between the three studies. The Vincent group found no evidence for the secretion of Wls on exosomes in the *Drosophila* wing imaginal disc and also found that blocking the formation of Wls-containing exosomes by interfering with the small GTPase Rab11 (Beckett *et al*., 2013; Koles *et al*., 2012) had no effect on Wg signaling in this tissue (Beckett *et al*., 2013). In contrast, the Boutros group did observe colocalization of Wls and Wg with punctate structures labeled with the exosomal marker CD63/GFP in the wing disc (Gross *et al*., 2012). Furthermore, they found that the inhibition of exosome secretion by knocking down the SNARE Ykt6 resulted in a reduction in Wg target gene expression and Wg loss of function phenotypes such as the loss of wing margin tissue. Taken together, these studies clearly show that Wnt proteins can be secreted on exosomes, but the *in vivo* role of this secretion mechanism in Wnt signaling needs to be further established.

How is the secretion of Wnt and Wls on exosomes related to the Golgi retrieval of Wls that we discussed in the previous section? An interesting possibility is that the exosome pathway acts in parallel to other release mechanisms. In such a scenario, Wnt binding may determine whether Wls is recycled or secreted through the exosome pathway. Thus, the pool of Wls that has released Wnt at the plasma membrane will be recycled, while Wnt-bound Wls may be shunted into the exosome pathway to generate a pool of Wnt with potential long-range signaling activity (Beckett *et al*., 2013; Gross *et al*., 2012).

Mechanisms that promote and control the diffusion of Wnt

Wnts mediate short- and long-range signaling

Wnt-producing cells can signal to directly neighboring cells but also to cells that are located at a distance. Short-range signaling occurs via direct cell–cell contact between the Wnt-producing cell and the signal-receiving cell. This type of Wnt-mediated cell communication is exemplified in the crypts of Lieberkühn of the small intestine, where differentiated Paneth cells directly present Wnt3 and other growth factors to sustain the adjacent stem cells (Sato *et al*., 2011). During embryonic development, Wnt signals are also communicated over longer distances to mediate tissue pattern formation. In these processes, Wnts act as morphogens by forming a gradient of extracellular protein to drive the activation of specific gene programs and cellular responses in a concentration-dependent manner (Strigini and Cohen, 2000; Vincent and Briscoe, 2001). The question of how the lipid-modified Wnts can be transported over long distances has been an intensely debated subject. Accumulating evidence suggests that the mechanisms of secretion and extracellular transport of Wnts differ between short- and long-range signaling (Bartscherer and Boutros, 2008; Coudreuse and Korswagen, 2007).

A number of factors that act at the interplay of proteins and lipids were implicated in long-range rather than short-range signaling. First, the association of secreted Wnts with lipoprotein particles promotes long-range signaling but leaves the expression of short-range target genes unaffected (Panakova *et al*., 2005). Of note, only a minor fraction of secreted Wnts was associated with lipoprotein particles in these studies (Panakova *et al*., 2005). These findings suggest that a small pool of Wnts destined for long-range signaling may require selective packaging.

A second factor implicated in the secretion and spreading of Wg in *Drosophila* wing discs is the membrane microdomain-forming component Reggie-1/flotillin-2 (Katanaev *et al*., 2008). Both Reggie-1/flotillin-2 and Reggie-2/flotillin-1 isoforms tightly bind the inner leaflet of the plasma membrane where they associate and polymerize to define specific microdomains in the plasma membrane (Otto and Nichols, 2011). A number of activities have been assigned to these proteins, including the regulation of endocytosis, signal transduction, and modulation of the cortical cytoskeleton. In Wnt-producing cells, Reggie-1 appears to be specifically required to generate and release a mobile form of Wnt that spreads efficiently into the tissue to mediate long-range target gene expression (Katanaev

et al., 2008). Reggie-1 also promoted secretion and spreading of the lipid-modified morphogen Hh, while other secreted factors such as Dpp and a GPI-linked form of GFP remained unaffected. The mechanism by which Reggie-1 activity contributes to the generation and secretion of Wnts remains unresolved. It is plausible that Reggie-1 facilitates trafficking or perhaps incorporation of Wg proteins in lipoprotein particles or exosomes. The level of conservation of this mechanism needs further investigation. As Reggie-1 or Reggie-2 homologs are absent in *C. elegans*, it will be interesting to determine if other proteins with similar microdomain-organizing activity may facilitate the extracellular mobility of Wnts in this organism.

Another protein called secreted Wingless-interacting molecule (Swim) was recently shown to promote long-range Wnt signaling (Mulligan *et al*., 2012) (Figure 1.1, step 5). Swim was identified in direct association with secreted Wg and significantly potentiated cellular responses to Wg. *Swim* RNAi experiments in *Drosophila* wing discs revealed no effect of Swim on the levels of Wg secretion or short-range signaling but demonstrated its involvement in the spreading of Wg and long-range target gene activation. How does Swim facilitate Wg mobility and signaling? The Swim protein shares a motif with members of the lipocalin family, which commonly facilitate the extracellular transport of hydrophobic proteins by shielding their lipid components (Flower, 2000; Ganfornina *et al*., 2000). Indeed, Swim binds Wg with high affinity and the interaction can be disrupted by palmitate in a dose-dependent manner, suggesting that Swim directly interacts with the lipid moiety on Wg (Mulligan *et al*., 2012). Together, these results lead to a model in which Swim maintains extracellular Wnt in soluble form by binding to and shielding its lipid tail. Once the Swim–Wnt complex reaches its target cells, the lipid tail of Wnt will need to be transferred to a groove in the Fz extracellular domain for a productive interaction (Janda *et al*., 2012). In agreement with this notion, Swim and Fz CRD were shown to compete for binding to Wg (Mulligan *et al*., 2012). The question whether Swim plays a part in the formation of Wg-containing lipoprotein particles or exosomes or perhaps represents another parallel pathway for Wnt transport remains unknown.

In conclusion, Wnt proteins destined for distant signaling require interactions with selective membrane microdomains as well as lipid-binding transport proteins. Thus, accumulating evidence suggests that packing of Wnts in specialized carriers is necessary for their long-range transport in extracellular space. Additional investigation is required to solve the question of whether the identified factors act in parallel or in sequential molecular steps that involve production, packing, release, and transport of Wnts.

Roles of HSPG in Wnt gradient formation

Wnt morphogens form gradients of extracellular protein that trigger concentration-dependent cellular responses during tissue patterning (Strigini and Cohen, 2000; Vincent and Briscoe, 2001). How these gradients are formed, shaped, and maintained is of fundamental interest but remains poorly understood (Lander, 2007). *Drosophila* 3rd instar larval wing imaginal discs have provided a powerful model for studying Wnt/Wg gradient formation. In this tissue, Wg secretion is confined to a narrow strip of cells at the dorsoventral (DV) boundary. Secreted, extracellular Wg proteins subsequently diffuse through the adjacent tissue to form a concentration gradient. High Wg concentrations close to the producing cells induce high-threshold target genes such as *senseless* (*sens*), while lower Wg concentrations farther away from the source induce low-threshold genes such as *dll* (Cadigan *et al*., 1998; Neumann and Cohen, 1997; Zecca, Basler, and Struhl, 1996).

The formation of a robust and stable morphogen gradient depends on an array of regulatory parameters, including rates of production, diffusion, retention, and endocytosis (Lander, 2007). Genetic approaches have identified HSPGs as major regulators of Wnt gradient formation and target gene activation in developing tissues (Hacker, Nybakken, and Perrimon, 2005; Lin, 2004) (Figure 1.1, step 6). HSPGs consist of a core protein that is heavily modified with heparan sulfate (HS), a type of glycosaminoglycan (GAG) (Sarrazin, Lamanna, and Esko, 2011). The highly negatively charged HS biopolymers can undergo an endless

number of alterations in number, length, and modification of the sugar chains, giving rise to an enormous diversity. Secreted HSPGs perform roles in the extracellular matrix and in secretory vesicles, while membrane-bound HSPGs were implicated in the formation of Hh, bone morphogenetic protein (BMP), fibroblast growth factor (FGF), and Wnt morphogen gradients (Hacker, Nybakken, and Perrimon, 2005; Lin, 2004; Sarrazin, Lamanna, and Esko, 2011).

Membrane HSPGs are subdivided in two families, called glypicans and syndecans. Glypicans are anchored to the cell membrane via glycosylphosphatidylinositol (GPI). Mammals carry six glypican genes, *Drosophila* has two, and *C. elegans* at least one. Sequence homology between family members is limited, but all glypicans share 14 Cys residues and 2–3 GAG attachment sites to membrane-proximal regions of the protein. Syndecans are type I transmembrane proteins that carry up to five GAG attachment sites that mainly contain HS chains. In mammals, four syndecan genes have been identified while invertebrates carry only one.

What is the evidence for the role of membrane HSPGs in Wg gradient formation and signaling? The initial identification of HSPGs in Wnt gradient formation came from genetic screens that searched for genes involved in embryonic segment polarity in *Drosophila* (Binari *et al*., 1997; Hacker, Nybakken, and Perrimon, 2005; Haerry *et al*., 1997; Lin and Perrimon, 1999; Luders *et al*., 2003; Selva *et al*., 2001). All of the identified genes in these studies encoded for enzymes or nucleotide sugar transporters involved in GAG biosynthesis (Hacker, Lin and Perrimon, 1997). Striking similarities in phenotypes between genes in HS biosynthesis and those of Hh and Wg pathways were found. As the Hh and Wg pathways are strongly interlinked through a positive feedback loop at this developmental stage, it has remained difficult to distinguish selective effects of HSPGs on the individual pathways. Subsequent studies in larval stage *Drosophila* wing discs provided conclusive evidence for a role of HS biosynthesis in Wg signaling and distribution. Mutations in genes involved in the cytosol-to-Golgi transport of GAG building blocks (*slalom, sll*), the transfer of sulfate groups to GAGs (*sulfateless, sfl*), or the assembly of the GAG backbone (*Ext* class of genes: *ttv*, *botv*, *sotv*) led to a decrease in levels of extracellular Wg and an abrogation of high-threshold Wnt target gene expression (Baeg *et al*., 2001; Han *et al*., 2004; Luders *et al*., 2003; Takei *et al*., 2004).

These findings clearly implicate HS biosynthesis in Wg signaling, but what HSPG core proteins are involved? Two HSPGs of the glypican family, Dally and Dlp, were placed centrally to Wg signaling events in *Drosophila* embryonic epidermis and developing wing discs (Baeg *et al*., 2001; Lin and Perrimon, 1999; Tsuda *et al*., 1999). The expression of Dally is positively regulated by Wg signaling, yielding highest levels close to the DV boundary in wing discs, where Wg is produced (Fujise *et al*., 2001; Han *et al*., 2005). Dlp, on the other hand, is negatively regulated by Wg signaling yielding low levels in a 7–10 cell-wide strip spanning the expression domain of Wg and increasing expression towards the tail end of the gradient (Han *et al*., 2005).

Dally binds and maintains Wg at the surface of cells within the range of the Wg gradient and shows genetic interaction with Wg signaling pathway components, and *dally* mutants display reduced extracellular Wg protein and wing margin defects (Han *et al*., 2005; Lin and Perrimon, 1999). The combined evidence suggests that Dally acts as a classical coreceptor that binds Wg and facilitates its interaction with Fz receptors (Figure 1.1, step 7), leading to the activation of signaling and rapid degradation of the complex (Franch-Marro *et al*., 2005; Han *et al*., 2005; Lin and Perrimon, 1999).

In contrast, the role of Dlp has puzzled researchers due to its biphasic activity in Wg signaling. In wing discs, Dlp expression promotes Wg activity in the tail end of the gradient where Wg ligands are low and reduces Wg activity close to the DV boundary where ligands are high (Franch-Marro *et al*., 2005; Hufnagel *et al*., 2006; Kirkpatrick *et al*., 2004; Kreuger *et al*., 2004; Yan *et al*., 2009). A number of studies have come up with explanations for this phenomenon. A consistent view is that Dlp captures Wg at the cell surface, prevents its degradation, and passes it on to neighboring cells, facilitating a unidirectional flow of Wg along the epithelial sheet to promote long-range signaling (Baeg *et al*., 2001; Franch-Marro *et al*., 2005; Hufnagel *et al*., 2006; Yan *et al*., 2009). But how does overexpressed Dlp inhibit short-range gene

expression when overexpressed near the Wg-producing cells? Recent work suggests that the biphasic activity of Dlp does not depend on its GPI anchor and does not involve shedding of Wg-bound Dlp from the cell surface, as suggested previously (Gallet, Staccini-Lavenant, Therond, 2008; Kreuger *et al*., 2004). Instead, Dlp either may compete with the Fz receptor for Wg binding or may retain Wg at the cell surface to promote its interaction with Fz, depending on the concentration ratio of Dlp, Wg, and Fz (Yan *et al*., 2009). Thus, the net flow of Wg is determined by the relative levels of ligand, receptor, and Dlp. Interestingly, a Dlp variant that lacks HS chains (DlpΔHS) showed a similar biphasic activity as the wild-type protein, suggesting involvement of the core protein (Yan *et al*., 2009). Indeed, the Dlp core protein interacted with Wg, and its modification with GAG chains further enhanced this interaction (Yan *et al*., 2009). These findings suggest that besides the evident contributions of HS chains, the core proteins enhance specificity to the roles of HSPG in different signaling pathways.

Tiki abrogates Wnt activity via cleavage of the Wnt N-terminus

A number of well-described secreted and membrane proteins can antagonize Wnt activity in the extracellular space, either by preventing productive Wnt-receptor interactions or by inhibiting Wnt receptor maturation (Cruciat and Niehrs, 2013) (see Chapter 13). Recently, a unique and highly conserved novel negative feedback mechanism was identified in Wnt signaling that involves the membrane-bound metalloprotease Tiki (Zhang *et al*., 2012). Tiki is a type I membrane protein that is induced by maternal Wnt signaling in the *Xenopus* head organizer region to drive anterior patterning via selective inhibition of the Wnt pathway. Tiki1 overexpression induced head enlargement (its name refers to the large-headed humanoid in Polynesian mythology), and knockdown led to anterior defects and loss of forebrain structures (Zhang *et al*., 2012).

Important mechanistic insight came from experiments in which Tiki was coexpressed with Wnt3a in mouse L cells. While Wnt3a was secreted normally from Tiki-expressing L cells, the protein showed faster electrophoretic migration, exhibited strongly impaired activity, and failed to bind its cognate receptors Fz and Lrp6 (Zhang *et al*., 2012). Edman amino acid sequencing revealed that Tiki induced cleavage of the eight most amino-terminal residues of Wnt3a (Figure 1.1, step 11). The purified Tiki ectodomain cleaved Wnt3a *in vitro* and its activity depended on bivalent metal ions, suggesting that Tiki is a metalloprotease. In phase-separation assays, the wild-type Wnt3a protein resided in hydrophobic detergent-solubilized fraction, while Tiki-modified Wnt3a partitioned exclusively in the aqueous phase (Zhang *et al*., 2012). Thus, by cleaving the Wnt N-terminus, Tiki alters the hydrophobicity of the Wnt protein. Strikingly, Tiki did not hamper lipidation of Wnt3a, suggesting that the enhanced Wnt3a solubility is mediated via conformational rearrangements. Indeed, Tiki-cleaved Wnt3a (as well as ΔN-Wnt3a) formed large soluble oligomeric complexes that were brought about via oxidation-mediated formation of intermolecular disulfide bonds (Zhang *et al*., 2012). How does the N-terminus of Wnt prevent oxidation–oligomerization? The recently solved structure of XWnt8 in complex with the XFz8 Cys-rich domain unfortunately does not reveal information on the orientation of the most N-terminal (and likely flexible) part of Wnt (Janda *et al*., 2012). Possibly, the N-terminus folds back onto the secreted Wnt protein to stabilize disulfide bonds in a conformation that allows exposure of the lipid tail. N-terminal cleavage by Tiki protease would drive the formation of alternative oligomeric conformations of Wnt that bury the lipid inside and render the complex hydrophilic. Interestingly, Tiki displays specificity for a number of different Wnts but fails to cleave Wnt11 (Zhang *et al*., 2012). This raises the question of how specificity of Tiki is regulated. Moreover, to what extent Wnts bound to the cell surface, exosomes, or lipoprotein particles are susceptible to Tiki cleavage and what is the role of Tiki in Wnt gradient formation remain important issues that await elucidation.

Conclusions and perspectives

The identification of specific cellular components that assist Wnt maturation and secretion

has delivered essential new insights into the mechanisms that underlie the generation of active Wnt proteins. Clearly, the lipid moiety on Wnts is a critical factor in the regulation of ER exit, Golgi-to-plasma membrane trafficking, release from the cell surface, extracellular spreading, and signaling of Wnts. During its intra- and extracellular journey, a number of regulatory proteins (including Wls, Swim, lipoproteins, Fz) bind and control Wnt activity in a lipid-dependent manner. These findings raise important new questions. How are lipidated Wnts transferred between cellular membranes, lipoprotein particles, and Fz receptors? How does Wls facilitate these events? Emerging evidence further suggests that distinct pools of extracellular Wnts exist. How different Wnt pools composed of exosomes, lipoproteins, or Swim-bound complexes contribute to Wnt gradient formation has yet to be solved. In addition, what are the regulatory mechanisms that control the activity of these Wnt subsets? Do these Wnt pools interact equally well with extracellular factors such as HSPGs, Tiki, and Fz receptors? A complete understanding of these issues will require the integration of genetic, cell biological, and biochemical approaches.

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