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REVIEW

# Mechanisms of intercellular Wnt transport

**Daniel Routledge and Steffen Scholpp***

## ABSTRACT

Wnt proteins are secreted glycoproteins that regulate multiple processes crucial to the development and tissue homeostasis of multicellular organisms, including tissue patterning, proliferation, cell fate specification, cell polarity and migration. To elicit these effects, Wnts act as autocrine as well as paracrine signalling molecules between Wnt-producing and Wnt-receiving cells. More than 40 years after the discovery of the Wg/Wnt pathway, it is still unclear how they are transported to fulfil their paracrine signalling functions. Several mechanisms have been proposed to mediate intercellular Wnt transport, including Wnt-binding proteins, lipoproteins, exosomes and cytonemes. In this Review, we describe the evidence for each proposed mechanism, and discuss how they may contribute to Wnt dispersal in tissue-specific and context-dependent manners, to regulate embryonic development precisely and maintain the internal steady state within a defined tissue.

**KEY WORDS: Cytoneme, Exosome, Secretion, Signal transduction, Wnt signalling, Wnt trafficking**

## Introduction

The Wnt signalling network comprises several signalling pathways, which are genetically and functionally conserved throughout metazoans (Loh et al., 2016). Wnt signalling regulates multiple processes crucial for embryogenesis and adult tissue homeostasis, including tissue patterning, cell polarity, migration and proliferation (Logan and Nusse, 2004). Aberrations in Wnt signalling can therefore lead to developmental defects and dysregulation of homeostatic processes, which control tissue size, organisation and function. Thus, Wnt signalling is implicated in a multitude of diseases, ranging from developmental disorders, such as Williams Syndrome, to several types of cancer, including colorectal, gastric and pancreatic cancers (Zhao et al., 2005; Chiurillo, 2015; Flanagan et al., 2017; Zhan et al., 2017).

Wnt proteins are a family of secreted glycoproteins, which share a conserved run of cysteine residues and an N-terminal signal sequence that targets them for secretion. In the extracellular matrix (ECM), Wnt proteins can act as autocrine and paracrine signalling proteins: Wnt ligands form gradients and act as morphogens to determine spatial identity and influence behaviour, such as gene expression, of target cells in a concentration-dependent manner (Gavin et al., 1990; Kiecker and Niehrs, 2001; Aulehla et al., 2003, 2008; Gao et al., 2011). To date, 13 Wnt gene families have been described: Wnt1-11, 16 and WntA, although the number of Wnt genes in individual species varies greatly. For example, all Wnt gene families (except Wnt9) are represented in the sea anemone, *Nematostella vectensis* (Stefanik et al., 2014). In protostomes, the number of Wnt genes ranges from about six in insects (seven in *Drosophila*) to 12 in the annelid worm *Platynereis dumerilii* (Swarup and Verheyen, 2012). However, a common feature among protostomes is the lack of the Wnt3 gene family (Janssen et al., 2010). In deuterostomes, all Wnt genes are present except the WntA gene family. In addition, the number of Wnt genes has increased following two whole-genome duplications (WGD), resulting in 19 Wnt genes in mice and humans (Miller, 2001). An additional WGD is observed in teleosts, increasing the number of Wnt genes even further to 27 in zebrafish (Duncan et al., 2015).

Transduction of Wnt signalling begins when these Wnt ligands bind receptors, including their cognate Frizzled (Fzd) receptor, at the cell membrane. Fzd receptors are seven-pass-transmembrane receptors with an extracellular cysteine-rich domain (CRD) and an intracellular PDZ-binding domain, of which there ten known paralogues in humans (Bhanot et al., 1996; Strutt et al., 2012). Fzd receptors activate the Wnt signalling network upon the binding of Wnt ligand to the CRD. This network is made up of several pathways, of which the best studied are the β-catenin-dependent pathway (Fig. 1A), and the β-catenin-independent/planar cell polarity (PCP) pathway (Fig. 1B) (Niehrs, 2012). These two pathways are primarily thought to act in a mutually repressive manner because both compete for common proteins, such as the scaffolding protein Dishevelled (Dvl) (Gao and Chen, 2010). The prevalence of a particular pathway therefore depends not only on the expression levels of specific Wnt proteins, but also of the Fzd receptors and specific co-receptors in a given cell or tissue at a given time point.

A commonly adopted view for Wnt signalling is that there are distinct populations of Wnt-producing and Wnt-receiving cells (Bartscherer and Boutros, 2008). How exactly Wnt is transported from one cell to the other is unclear, as Wnts are hydrophobic as a result of post-translational lipid modifications and are thus unlikely to diffuse freely (Willert et al., 2003; Takada et al., 2006). In this Review, we discuss the intracellular and intercellular transport of Wnt, focusing on proposed mechanisms that mediate the extracellular transport of Wnt proteins (summarised in Table 1). This combined knowledge of Wnt intercellular transport will improve our understanding of how Wnt morphogen gradients are formed during developmental processes and how Wnt dispersal is achieved in tissue homeostasis.

## Wnt secretion

### Wnt ligand processing

Following synthesis, Wnt proteins are processed in the endoplasmic reticulum (ER) where they undergo various post-translational modifications (PTMs). Except for the distantly related *Drosophila* WntD, all analysed Wnt proteins are lipid-modified through mono-palmitoylation of a conserved serine residue (Takada et al., 2006; MacDonald et al., 2014). This modification requires the O-acyltransferase Porcupine (Porcn), an ER-localised enzyme that catalyses the transfer of palmitoleic acid onto Wnts (Kadowaki et al., 1996). Varying patterns of other PTMs, such as glycosylation,

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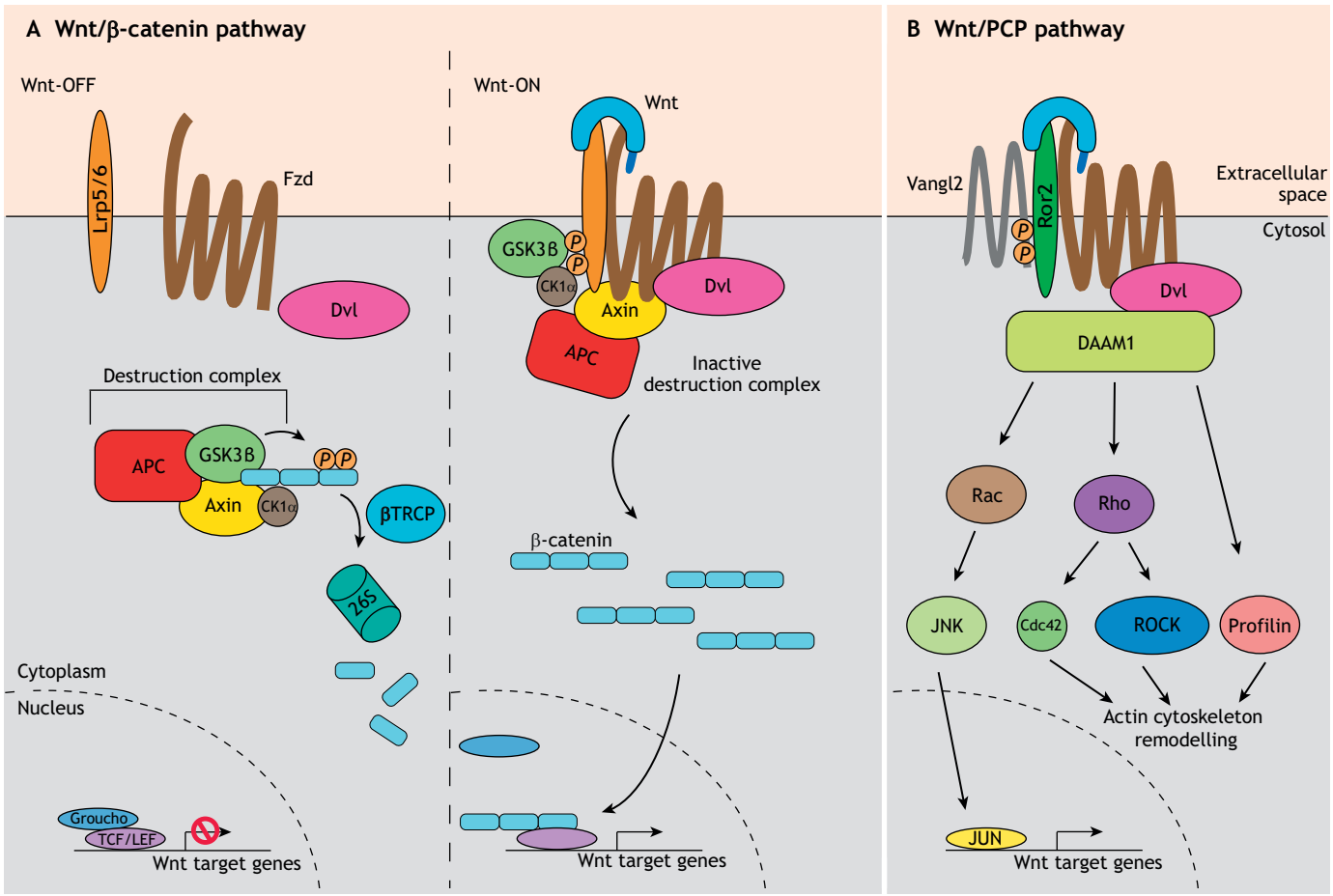


**Fig. 1. Wnt signalling network.** The Wnt signalling network contains several branches from which the β-catenin-dependent pathway and the planar cell polarity (PCP) pathway are best described. (A) In the β-catenin-dependent pathway, β-catenin undergoes continuous turnover in the absence of Wnt signals by the destruction complex (Wnt-OFF). In this state, Wnt target genes are suppressed by Groucho and TCF/LEF transcription factors. Upon Wnt binding to canonical Fzd receptors and the co-receptor Lrp5/6, a ligand-receptor complex called the 'signalosome' is formed. This causes the intracellular recruitment of Dvl and components of the destruction complex. Recruitment to the plasma membrane inhibits the formation of a functional destruction complex and thus prevents the degradation of β-catenin, permitting its cytosolic accumulation. β-Catenin subsequently translocates to the nucleus, where it binds with TCF/LEF transcription factors to inhibit their DNA binding. Wnt target genes, such as cyclin D1 and *Myc*, are disinhibited to control cell fate acquisition and proliferation. (B) In the β-catenin-independent/PCP pathway, Wnt binding to non-canonical Fzd receptors, along with co-receptors such as Ror2, induces actin polymerisation through activation of cytoskeletal regulators. These include the small GTPases Rho, Rac and Cdc42, which promote elongation or branching of actin filaments. These drive extension of the cell membrane in the form of lamellipodia and filopodia to regulate cell polarity and migration. 26S, 26S proteasome holoenzyme; APC, adenomatous polyposis coli; CK1α, casein kinase 1α; Daam1, dishevelled associated activator of morphogenesis 1; Dvl, dishevelled; GSK3, glycogen synthase kinase 3; Fzd, Frizzled; Ror2, receptor-tyrosine kinase-like orphan receptor 2; βTRCP, ubiquitin ligase SCF.

distinguish Wnt proteins and their concomitant signalling properties (Yamamoto et al., 2013). For example, Wnt1 harbours four *N*-linked glycosylations, whereas Wnt3a only has two. Furthermore, the palmitoleic acid lipid group on Wnts is indispensable for the secretion and function of Wnt proteins, and gives the protein hydrophobic properties. For example, several studies have reported that deletion or inhibition of Porcn results in the aberration of Wnt signalling and retention of Wnt in the ER (Barrott et al., 2011; Biechele et al., 2011). Analogous results have been observed using a S209A substitution in Wnt3a, which prevents Porcn-mediated acylation at this site and also results in its retention in the ER (Takada et al., 2006). Furthermore, recent observations from the crystal structure of the Fzd7 CRD bound to a fatty acid has revealed that the palmitoleic lipid adduct binds to a U-shaped lipid-binding cavity of the Fzd7 dimer. Fzd5 and Fzd8 have similar architectures, including a dimeric arrangement of the CRD, suggesting a common model for how Wnt binds Fzd receptors via the fatty acid modification (Nile et al., 2017).

### Intracellular trafficking

The discovery of the intracellular Wnt chaperone Wntless (Wls, also known as Evi/Sprinter) has provided clues for the role of PTMs in the process of secretion, as Wls binds to Wnt through its palmitoleic acid moiety to transport Wnts from the ER via the Golgi to the plasma membrane (Fig. 2) (Yu et al., 2014). Indeed, loss of Wnt palmitoylation prevents Wls-Wnt interaction (Bänziger et al., 2006; Bartscherer et al., 2006). In addition, depletion of Wls disrupts Wnt signalling in HEK293T cells by preventing Wnt3a reaching the cell surface or being secreted into the culture medium (Bänziger et al., 2006). Wnt proteins also stabilise Wls levels, as *Wnt3a* expression in HEK293T cells results in increased levels of Wls protein. Interestingly, this accumulation is not accompanied by an increase in *Wls* mRNA levels, suggesting that Wnt signalling does not transcriptionally regulate *Wls*. As treatment with proteasome inhibitors increases Wls protein levels in the absence of Wnt3a, and levels of poly-ubiquitylated Wls decreases in the presence of Wnt3a, it has been suggested that Wnt proteins aid stabilisation

Development (2019) 146, dev176073. doi:10.1242/dev.176073

**Table 1. A summary of mechanisms of Wnt protein transport observed in different organisms**

| Transport mechanism | Wnt proteins | Organism/tissue/cell | References |
|---|---|---|---|
| Wnt-binding chaperones | Wg (Swim) | *Drosophila* wing imaginal disc | Mulligan et al., 2012 |
| | Wnt3a, Wnt5a (afamin) | Human (HEK293 cells) | Mihara et al., 2016 |
| HSPGs | Wg | *Drosophila* embryos | Baeg et al., 2001; Chang and Sun, 2014 |
| | Wnt11 | Zebrafish, *Xenopus* embryos | Topczewski et al., 2001; Ohkawara, 2003 |
| Lipoproteins | Wg | *Drosophila* wing epithelium | Panáková et al., 2005 |
| | Wnt5a | Mouse choroid plexus epithelial cells (*in vivo*) | Kaiser et al., 2019 |
| Exosomes | Wg | *Drosophila* neuromuscular junction and wing disc | Korkut et al., 2009; Gross et al., 2012 |
| | Wnt3a | Human (HEK293 cells) | Gross et al., 2012 |
| Cytonemes | Wnt2b | *Xenopus* fibroblasts (*in vitro*) | Holzer et al., 2012 |
| | Wnt8a | Zebrafish embryos, human (gastric cancer cells) | Stanganello et al., 2015; Mattes et al., 2018 |

of Wls by preventing its proteasome-dependent degradation (Glaeser et al., 2018). In addition to Wls, it has been suggested that the *Drosophila* p24 cargo adaptor protein Opossum (Opm) shuttles proteins, including the *Drosophila* Wnt orthologue, Wingless (Wg), across the ER-Golgi interface (Buechling et al., 2011). Thus, the chaperone-like proteins Wls and Opm have both been proposed to mediate ER-to-Golgi transport of Wnt proteins.

Following delivery of Wnt to the plasma membrane, Wls is thought to be endocytosed and recycled in the Wnt-producing cell via the retromer complex: a multi-protein complex that redirects Wls away from the lysosomal degradative pathway and back to the ER. Here, it can bind to newly synthesised Wnt proteins and traffic them back to the membrane (Fig. 2) (Belenkaya et al., 2008; Yu et al., 2014). This model explains how inhibiting retromer function in Wnt-producing cells attenuates Wnt secretion by preventing the recycling of Wls, and thus trafficking of Wnt to the cell surface (Franch-Marro et al., 2008).

#### Wnt release

Once Wnt has reached the plasma membrane, the question of how Wnt is released from Wls and secreted to fulfil its paracrine function remains highly debated. Long-range, free diffusion of the lipid-modified Wnt proteins in the aqueous extracellular space seems unlikely, because of their hydrophobic nature. Indeed, Wnt proteins form aggregates in the ECM unless stabilised by detergents or serum (Fuerer et al., 2010). Thus, without assistance, Wnt signalling is restricted to autocrine and probably juxtacrine signalling. It has been suggested, however, that short-range signalling is sufficient for growth and development in several tissues. One such report highlights that short-range transport of Wnt proteins can be achieved without secretion in the intestinal crypt. Here, Wnt protein can be detected away from *Wnt*-expressing cells because it travels in a cell-bound manner through cell divisions (Farin et al., 2016). In addition, *Drosophila* mutants with a membrane-tethered form of Wg are viable despite attenuated Wg gradients. However, membrane-tethered Wg mutants develop slightly smaller wings with a delay. Thus, it has been suggested that early *wg* expression is sufficient to induce persistent target gene expression and that long-range signalling supports, but is not essential for, later stages of wing growth and development by promoting cell proliferation (Alexandre et al., 2014).

The suggestion that long-range Wg signalling is dispensable for tissue patterning contradicts our previous understanding of Wg acting as a morphogen. For example, in *Drosophila*, extracellular Wg protein has been detected up to 11 cell diameters from the producing cells, and Wg target genes are expressed up to 20 cell diameters away (Zecca et al., 1996; Neumann and Cohen, 1997; Chaudhary and Boutros, 2018 preprint). Supporting these observations, Wg has been shown to control wing growth through long-range activation of target genes, such as *Distal-less* (*Dll*) and *vestigial* (*vg*). Indeed, ectopic expression of *wg* increases expression of these genes, which results in overgrowth of the wing pouch (Neumann and Cohen, 1997). One possible explanation for this discrepancy could be that membrane-associated Wg is transported over long distances by alternative transport mechanisms, as discussed below. However, the requirement for long-range signalling during embryogenesis remains to be clarified.

What determines whether a Wnt protein is destined for short- or long-range dispersal? In *Drosophila*, this is thought to be regulated in a polarised manner, as apical and basolateral secretion of Wnt proteins can produce short- and long-range gradients, respectively (Bartscherer and Boutros, 2008; Chaudhary and Boutros, 2018 preprint). For example, long-range extracellular Wg gradients form on the basolateral surface of the wing disc (Strigini and Cohen, 2000). In polarised human epithelial cells, Wnt3a is secreted basolaterally in a Wls-dependent manner. In addition, secretion of Wnt3a is also attenuated by depletion of Clathrin, a protein that forms a major role in vesicle formation, which suggests that endocytosis in involved in Wnt3a secretion (Yamamoto et al., 2013). Concurrent with this notion, in *Drosophila* shibire mutants, which have impaired endocytosis due to mutations in the Dynamin gene, Wg-producing cells accumulate Wg protein (Strigini and Cohen, 2000). Conversely, Wnt11 is secreted apically and its secretion is not affected by Wls or Clathrin depletion, which suggests a different mechanism controls Wnt11 secretion (Yamamoto et al., 2013). The secretory routes for individual Wnt proteins might be determined by differences in post-translational glycosylation of Wnt3a and Wnt11 (Yamamoto et al., 2013). A proposed explanation for these polarised phenotypes is Wnt transcytosis, whereby Wnt ligands are first presented at the apical membrane to mediate short-range signalling, before being re-endocytosed, packaged into endosomes and transported to the basolateral membrane for secretion (Yamazaki et al., 2016). Indeed, Wg has been observed on the apical membrane before being re-endocytosed in the secreting cells (Pfeiffer et al., 2002). From here, Wnt secretion is thought to mediate long-range signalling and gradient formation. Several mechanisms to explain this long-range spreading of Wnt have been proposed, including Wnt-binding chaperone proteins, lipoproteins, exosomes and cytonemes, as we discuss below (Port and Basler, 2010; Stanganello and Scholpp, 2016).

## Wnt carriers

### Protein chaperones

A common mechanism utilised by cells to shield hydrophobic structures or proteins from the aqueous environment is through

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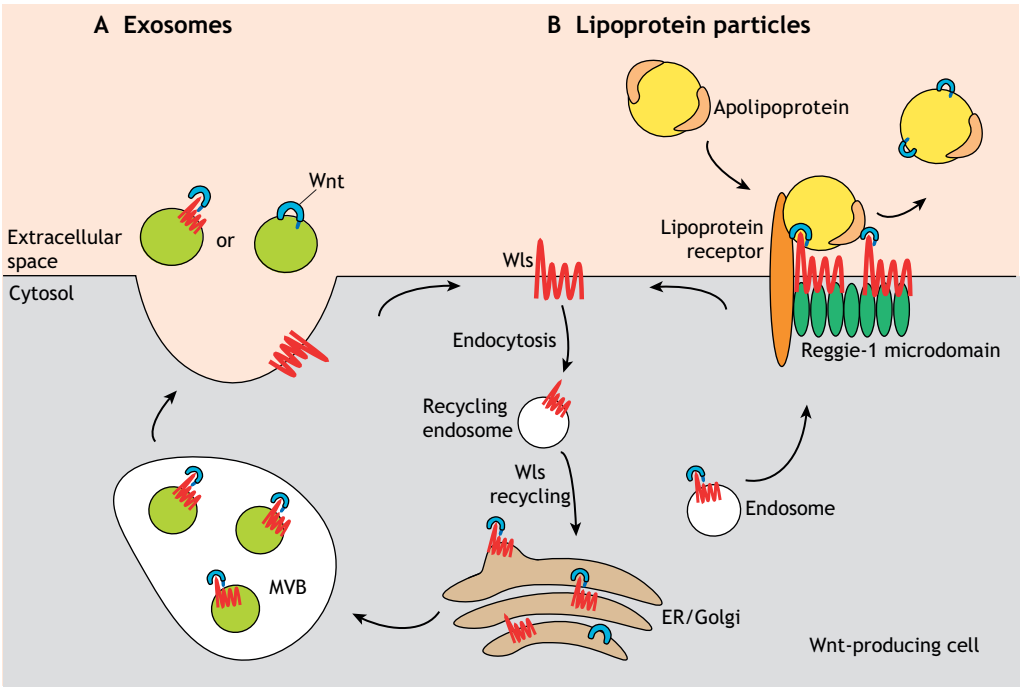


**Fig. 2. Trafficking of Wnt on extracellular vesicles.** (A) The Wnt-Wls complex traffics through the ER and Golgi before being loaded onto exosomes, which are contained within multi-vesicular bodies (MVBs). Subsequent fusion of MVBs with the plasma membrane results in the release of Wnt-bearing exosomes. Wnt is associated with exosomes either bound with Wls, or they dissociate and Wnt tethers to the exosomal membrane via its lipid moiety. In the latter, Wls is then re-endocytosed and recycled back to the ER/Golgi. (B) Lipoproteins are mainly synthesised in the liver/fat body. After secretion, lipoproteins interact with lipoprotein receptors, such as SR-BI/II, at the cell surface, which are localised to Reggie-1 microdomains. The Wnt-Wls complex is trafficked to these domains, where Wnt and Wls dissociate and Wnt is loaded onto the lipoprotein, presumably tethered to the membrane via its lipid moiety. Wls is then re-endocytosed and recycled back to the ER/Golgi.

binding to other proteins, which protect these hydrophobic regions, aid their stabilisation and improve solubility. This is exemplified by intracellular binding proteins, such as fatty acid-binding proteins (FABPs) and retinol-binding protein (RBP), which help the solubilisation, transport and secretion of fatty acids and retinol, respectively (Ronne et al., 1983; Storch et al., 1996). Given the hydrophobic nature of Wnts, it is therefore conceivable that Wnt proteins could be transported through a similar mechanism.

One family of proteins known to bind to Wnts are secreted Frizzled-related proteins (sFRPs) (Hoang et al., 1996). sFRPs are known to modulate Wnt signalling, and this is thought to be through interaction with Wnt receptors or sequestration of Wnt proteins (Fig. 3) (Üren et al., 2000; Galli et al., 2006). However, the role of sFRPs in modulating Wnt signalling is unclear; sFRPs were first reported as Wnt inhibitors (Leyns et al., 1997), but increased expression of sFRPs can both inhibit and augment Wnt signals in context- and concentration-dependent manners (Üren et al., 2000; Houart et al., 2002; Xavier et al., 2014). In *Xenopus* embryos, sFRPs have been shown to enhance the diffusion of Wnt8 and Wnt11 by forming a complex (Mii and Taira, 2009). Therefore, sFRPs might aid the transport of Wnt, but at high concentrations, sFRPs could also outcompete Wnt receptors to inhibit Wnt signalling. Exactly how sFRPs differentially modulate Wnt signalling is yet to be determined. Recently, a lipocalin protein in *Drosophila* termed Secreted Wg-interacting Molecule (Swim) was suggested to facilitate long-range Wg transport by maintaining its solubility in the ECM and thus aiding its transport to Wg-receiving cells (Mulligan et al., 2012). However, no vertebrate homologue of Swim has been identified and further follow-up genetic studies would be necessary to assess the function of Swim in detail.

In humans, the glycoprotein afamin has been unexpectedly reported to bind to Wnt (Mihara et al., 2016). Afamin is a member of the serum albumin family group of binding proteins, which display an affinity for a wide variety of poorly soluble molecules, including lipid-modified proteins that interact via a hydrophobic binding pocket (Naschberger et al., 2017). Although afamin is renowned for its vitamin E-binding capabilities (Dieplinger and Dieplinger, 2015), afamin has been co-purified with Wnt3a from HEK293 cells and has been shown to enhance Wnt3a secretion in a dose-dependent manner, potentially by enhancing its solubility (Mihara et al., 2016). Following these findings, afamin was shown to associate with, and enhance the secretion of, 12 different Wnt proteins *in vitro* (Mihara et al., 2016).

Crucial to its function as a paracrine signalling factor, Wnt3a maintains its biological activity when in complex with afamin, which improves its solubility (Mihara et al., 2016). The hydrophobic pocket of afamin is suspected to bind Wnt proteins through their shared palmitoleic acid modification; Naschberger and colleagues (Naschberger et al., 2017) computationally modelled the Wnt3a-afamin complex, based on the crystal structure of *Xenopus* Wnt8 bound to the CRD of Frizzled 8 (XWnt8-Fzd8-CRD). Here, the hydrophobic cavity of Fzd8-CRD accommodates the S187 palmitoleic acid of XWnt8 (Janda et al., 2012). Indeed, the resulting model describes the S209 palmitoleic acid of Wnt3a to be central to its binding to afamin (Naschberger et al., 2017). Together, these findings highlight a novel role for afamin in extracellular Wnt transport. However, afamin is primarily expressed in the liver and transported in the blood in vertebrates. Although its role in the context of *in vivo* Wnt signalling is yet to be elucidated, it is unlikely to represent an evolutionarily conserved mechanism of Wnt

Development (2019) 146, dev176073. doi:10.1242/dev.176073

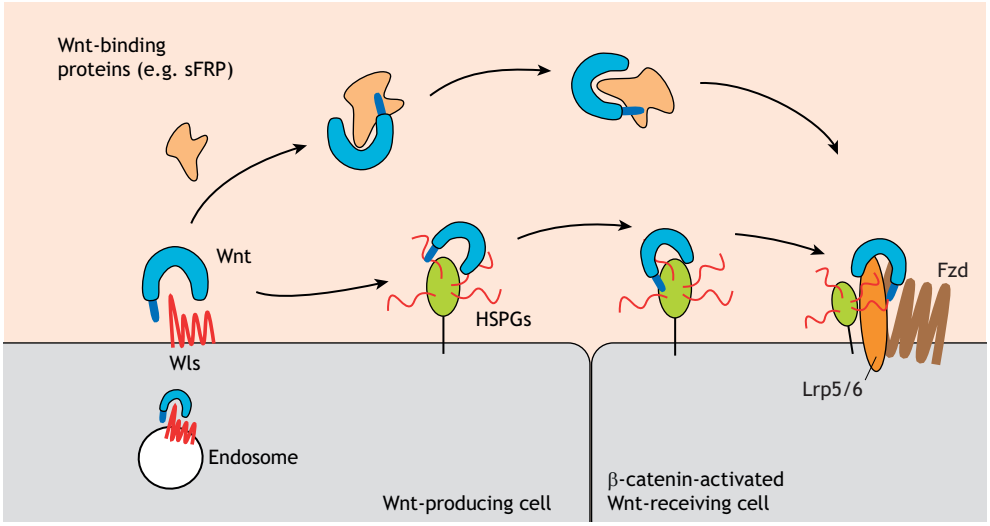


**Fig. 3. Facilitated diffusion of Wnt.** Following its dissociation from Wls at the cell surface, Wnt-binding proteins, such as sFRPs and afamin, bind to Wnt via its hydrophobic lipid moieties to increase its solubility and act as a chaperone protein to enhance its extracellular diffusion. Wnt interacts with cell surface HSPGs, such as Dally and Dally-like (Dlp), which stabilise the Wnt protein and may improve its lateral diffusion.

dispersal, because invertebrates do not express albumin family proteins (Baker, 1998).

There is some evidence, however, that Wnt proteins may diffuse freely in the extracellular space without protein chaperones. A recent study reports free extracellular dispersal of the Wnt orthologue EGL-20 in *Caenorhabditis elegans* (Pani and Goldstein, 2018). However, how diffusion is achieved is unclear. Using fluorescence recovery after photobleaching (FRAP), fluorescently tagged EGL-20 could be visualised in photobleached areas within 30 s, which is consistent with free extracellular spreading *in vivo* (Muller et al., 2013). Whether this dispersal is achieved through stabilisation by Wnt/EGL-20-binding proteins or ECM components remains to be determined, and studies to investigate EGL-20-binding proteins could provide clarity on how EGL-20 is stabilised to achieve free extracellular dispersal (Pani and Goldstein, 2018).

### The role of heparan sulphate proteoglycans

Although the free diffusion of Wnt proteins is largely disputed, Wnt proteins can be stabilised to prevent aggregation and thus facilitate spreading through the ECM. One proposed mechanism involves interactions of Wnt proteins with heparan sulphate proteoglycans (HSPGs), a component of the ECM (Fig. 3). HSPGs bind to a plethora of ligands and are traditionally thought to serve as co-receptors to promote binding of ligands to their receptors. In addition, HSPGs have also been shown to interact with many morphogens (Kirkpatrick and Selleck, 2007). Indeed, HSPGs are thought to enhance Wnt spreading through ligand stabilisation. For example, in *Drosophila*, overexpression of the glypican *dally-like* (*dlp*) leads to sequestration of Wg at the cell surface (Baeg et al., 2001). Conversely, Wg is not observed on the surface of cells expressing sugar-deficient HSPGs. HSPGs are also suggested to facilitate binding of Wg to its receptor, because overexpression of *wg* can rescue the phenotypes of *sugarless* mutants, which lack an enzyme involved with proteoglycan synthesis (Hacker et al., 1997). This function of HSPGs appears to be conserved outside of *Drosophila*; the zebrafish HSPG-encoding gene, *glypican 4* (also known as *knypek*), regulates gastrulation events through potentiation of Wnt11 signalling (Topczewski et al., 2001). Furthermore, in *Xenopus* embryos glypican4 interacts with Wnt11, and the glycosyltransferase XEXT1, involved with heparan sulphate synthesis, is necessary for Wnt11-induced axis formation (Ohkawara, 2003; Tao et al., 2005). Together, these findings implicate HSPGs as mediators of Wnt signalling and indicate that HSPGs presumably also influence spreading.

HSPGs may also aid the delivery of Wnt-bearing structures through interactions with transport machinery. For example, HSPGs are thought to act as bulk endocytosis receptors and thus help the delivery of lipoproteins and exosomes to target cells (Christianson and Belting, 2014). In *Drosophila*, the HSPGs Dally and Dlp aid the recruitment of Hh-positive lipoproteins to wing disc cells through direct interactions with lipophorins (Eugster et al., 2007). These interactions are thought to be mediated through HSPG sugar moieties, as altering the composition of heparin sulphate glycosaminoglycans (GAGs) attenuates their affinity for and clearance of lipoproteins in hepatocytes (Olsson et al., 2001; Stanford et al., 2010). HSPGs may also interact with low density lipoprotein receptors on the surface of lipoproteins, which have been shown to co-immunoprecipitate in mouse embryonic fibroblasts (MEFs) (Wilsie and Orlando, 2003). Similarly, endocytic uptake of exosomes is thought to be dependent on HSPGs, as inhibition of proteoglycan synthesis attenuates exosome uptake in glioblastoma cells (Christianson et al., 2013). Analogous results have been seen after treatment with free heparan sulphate chains, which compete with HSPGs for exosome binding, although binding interactions for this are not known (Christianson et al., 2013). Together, these findings highlight a potential role for HSPGs as endocytic receptors for extracellular vesicles. In this manner, HSPGs may aid the internalisation of Wnt-bearing lipoproteins and exosomes in target cells.

The function of HSPGs may also allow the formation of long-range gradients of Wnt proteins without the need for ligand mobilisation. In chick development, Wnt ligands can be loaded onto migrating neural crest cells that deliver their message at a distance (Serralbo and Marcelle, 2014). To improve the delivery process, neural crest cells express glypican 4, which acts in trans to deliver the Wnt ligand to the receiving cells in the somites. Therefore, by

Development (2019) 146, dev176073. doi:10.1242/dev.176073

mobilising the source cells, one can achieve a long-range signalling gradient in some tissues.

### Lipoproteins

Lipoproteins are a class of extracellular membrane vesicle that function as a crucial intercellular communication mediator regulating the exchange of proteins and genetic materials between donor and surrounding cells. The first evidence that Wnts may be transported via lipoproteins came from the colocalisation of membrane-tethered GFP with Wg-containing vesicles, thought to derive from the basolateral membrane of Wg-producing cells (Greco et al., 2001). More recently, these structures have been identified as lipoproteins; globular vesicles typically used for transporting hydrophobic lipids and proteins. Lipoprotein particles are of interest in Wnt signalling because Wg co-purifies with lipophorins, the *Drosophila* homologue of lipoproteins. In addition, Wg colocalises with lipophorins in the developing wing epithelium and RNAi knockdown of lipophorins shortens Wg gradients, as measured by expression of target genes in Wg-receiving cells. Analogous results have been seen for Hedgehog (Hh) signalling, another lipid-modified morphogen, which indicates that lipoprotein particles are a common mechanism for long-range morphogen signalling (Panáková et al., 2005).

This concept has also been observed in a mammalian context, where Wnt3a associates with lipoproteins *in vitro* in the media of mouse fibroblasts. However, when grown in media containing delipidated foetal calf serum, which lack lipoproteins, Wnt3a is not detected in the media. The addition of high-density lipoproteins (HDLs), but not low-density lipoproteins (LDLs), leads to the release and increased levels of Wnt3a in the media, suggesting that Wnt3a can be loaded onto exogenous HDLs (Neumann et al., 2009). Furthermore, Wnt5a is produced in the murine choroid plexus (CP) and is required for morphogenesis of the dorsal hindbrain. Recently, it was shown that Wnt5a colocalises with lipoproteins in CP epithelial cells and target hindbrain progenitors at the ventricle, which express Wnt signalling components as well as receptors for lipoprotein particles (Kaiser et al., 2019). Although a mechanism explaining how Wnt may be loaded onto lipoproteins is yet to be determined, lipoprotein-mediated transport seems to be an important Wnt transport mechanism in the context of the cerebrospinal fluid.

In *Drosophila*, Wg localises to Reggie-1-positive microdomains at the plasma membrane. Reggie-1 (also known as Flotillin 2) is an acylated, membrane-bound scaffolding protein, which can localise and oligomerise at sphingolipid-rich lipid microdomains (Langhorst et al., 2007). Although the exact function of Reggie-1 remains to be clarified, in the context of Wg signalling it has been suggested to aid the secretion of Wg. Indeed, *Reggie-1* overexpression or knockdown expands or reduces extracellular Wg gradients, respectively (Katanaev et al., 2008). In addition, Wg has been observed to partially colocalise with Reggie-1 (Katanaev et al., 2008). One hypothesis is that Reggie-1 microdomains serve as ‘dating points’ to which lipoprotein receptors and Wnt/Wg colocalise; permitting the loading of Wnt/Wg onto exogenous lipoproteins (Fig. 2B) (Solis et al., 2013).

Alternatively, some cell types are capable of lipoprotein synthesis. For example, Wnt3a secretion via endogenous lipoprotein particles is observed *in vitro* in intestinal epithelial cells. Here, Wnt3a co-precipitates with newly synthesised apoB100, a poorly lipidated apolipoprotein associated with LDLs (Neumann et al., 2009). Concurrent with reports that lipoproteins are basolaterally derived, these endogenous lipoproteins are also observed on the basolateral side, whereas exogenous HDLs and the lipoprotein receptor SR-BI/II are predominantly localised at the apical surface of polarised epithelial cells (Reboul et al., 2006; Neumann et al., 2009). This could suggest two different lipoprotein-based mechanisms for Wnt secretion, whereby different Wnt proteins may be loaded onto exogenous or endogenous lipoproteins, which provide alternative secretory routes. This concept is supported by the observation that Wnt3a and Wnt11 ligands are secreted basolaterally and apically, respectively, and that they are both differentially regulated (Yamamoto et al., 2013). However, whether Wnts maintain biological activity when lipoprotein-bound has not been clarified, and a role for lipoproteins in Wnt transport *in vivo* is yet to be examined.

### Exosomes

Supporting the concept of Wnt transport via extracellular vesicles, exosomes have also been proposed to mediate extracellular Wnt transport. Although they are conceptually comparable mechanisms (shielding hydrophobic proteins in a membranous vesicle), exosomes differ from lipoproteins in their composition and biosynthesis. Exosomes are double-membrane, cell-derived vesicles that form during the maturation of early endosomes into multivesicular bodies (MVBs), in which they are contained. As they are trafficked through the endosomal compartments, exosomes are loaded with cargo proteins and secreted from cells through the fusion of MVBs with the plasma membrane (Fig. 2A) (Hessvik and Llorente, 2018). Exosomes then move through the ECM to deliver proteins to other cells; probably mediating intercellular communication.

A role for exosomes in transporting Wnt proteins was first reported in the *Drosophila* neuromuscular junction (Korkut et al., 2009). Here, Wg is carried across the synaptic cleft by Wls-containing exosomes to influence synaptic growth, function and plasticity. This observation is supported by a study that showed Wnt3a can localise with exosomes from HEK293 cells (Gross et al., 2012). By using TSG101 protein as an exosomal marker, immunoblot analysis of lysates of Wnt-expressing cells revealed the presence of Wnt3a and Wnt5a in the exosomal fractions. Furthermore, *in vivo* staining of the *Drosophila* wing disc revealed colocalisation of Wg and the exosomal marker CD63-GFP in both intracellular MVBs and the extracellular space, although this was only a fraction of the total Wg staining (Gross et al., 2012). The significance of exosome-mediated transport is becoming evident in a variety of contexts. In CNS injury, for example, fibroblast-derived exosomes promote axonal regeneration by inducing re-localisation of neuronal Wnt10b to lipid rafts, which promotes CNS repair through mTOR pathway activation (Tassew et al., 2017). Conversely, the presence of exosomes in cancer often correlates with poor prognosis; there is evidence of stromal cells utilising exosomes to transport pro-tumorigenic factors, such as growth factors, microRNAs (miRNAs) and Wnt proteins (Halvaei et al., 2018; Hu et al., 2018).

Interestingly, the Wnt chaperone Wls has also been found in MVBs, where it colocalises with Wnt and the exosomal/MVB markers CD81 and TSG101 (Gross et al., 2012). An essential maturation step of MVBs is endosomal acidification, which can be blocked by the V-ATPase inhibitor bafilomycin A1 (Clague et al., 1994). Inhibiting endosomal acidification (and thus MVB maturation) causes intracellular accumulation of the Wls-Wnt3a complex (Coombs et al., 2010). However, the persistence of the Wls-Wnt complex in exosomes is unclear; Wls and Wnt are separated in MVBs and are suggested to be secreted on different exosomes, because only 10% of total Wls and Wg protein colocalises extracellularly (Gross et al., 2012). Furthermore, in

Development (2019) 146, dev176073. doi:10.1242/dev.176073

*Drosophila* embryos, Wg remains tightly associated with producing cells and is endocytosed from the plasma membrane (Pfeiffer et al., 2002). These findings are consistent with the proposed retromer-dependent recycling of Wls in Wg-producing cells, which requires its endocytosis (Fig. 2) (Port et al., 2008). However, Wls has been observed on secreted exosomes *in vivo* in the neuromuscular junction of *Drosophila*, where bi-directional Wg signalling (i.e. activation of Wg signalling in both pre- and post-synaptic neurons) modulates synaptic structure and function (Ataman et al., 2008; Koles et al., 2012). Although this breaks from the classical 'Wnt-producing and Wnt-receiving' model introduced above, neurons may require bi-directional signalling to regulate synaptic stability and activity, a process that is regulated by both anterograde and retrograde signalling (Haghighi et al., 2003). Although the point at which Wls and Wnt/Wg dissociate is disputed, there is substantial evidence that Wnt is transported on exosomes. Clarifying the persistence of Wls-Wnt interactions on exosomes is crucial to gaining a molecular understanding of this process.

### Cytonemes

First described in *Drosophila* wing imaginal disc, cytonemes represent a subset of specialised filopodia capable of transporting signalling components to neighbouring cells (Ramírez-Weber and Kornberg, 1999). In the wing imaginal disc, cytonemes are primarily associated with the transport of morphogens, such as fibroblast growth factor (FGF), Hh and Decapentaplegic (Dpp), which has been suggested to aid formation of gradients pivotal to correct tissue patterning during development (Roy et al., 2014; González-Méndez et al., 2017). Wg is also a key morphogen in *Drosophila* development; particularly in wing imaginal disc formation, as loss of Wg signalling results in loss of wing structures (Sharma and Chopra, 1976). Although transport of Wg on cytonemes has not been directly observed, its receptor Fzd is present on the cytonemes of wing disc myoblasts. Here, Wg forms a complex with Fzd and the cytoneme retracts towards the Wg-receiving cell in a retrograde manner (Huang and Kornberg, 2015). Experiments in *Drosophila* and cell culture revealed that signalling molecules could be disseminated by cell protrusions (Mattes and Scholpp, 2018). Indeed, recent high-resolution imaging experiments in zebrafish confirmed such a novel and unexpected mechanism for the extracellular transport of Wnt (Stanganello et al., 2015). In particular, cytonemes have been demonstrated to be fundamental in Wnt trafficking in vertebrates (Stanganello et al., 2015).

Cytoneme-mediated transport of Wnt has been most extensively studied in vertebrate organisms where, unlike in *Drosophila*, the ligand (Wnt), rather than the receptor (Fzd), is transported via cytonemes to the target cells (Stanganello et al., 2015). For example, Wnt2b-EGFP and Wnt8a-GFP have been visualised on cell protrusions in *Xenopus* and zebrafish embryos, respectively (Holzer et al., 2012; Luz et al., 2014). In the latter case, Wnt8a is transported on the tips of Cdc42/N-Wasp-positive cytonemes to influence tissue patterning in the neural plate by inducing Wnt signalling in receiving cells (Fig. 4) (Stanganello et al., 2015).

The formation of Wnt-positive cytonemes is driven by the expression of Wnt genes. Akin to the models as mentioned above, Wnt is proposed to traffic from the ER to the plasma membrane with its chaperone Wls (Gradilla et al., 2018). Cytoneme formation is driven by activation of cytoskeletal regulators (such as the small GTPases Rho, Rac1 and Cdc42), which drive actin polymerisation (Spiering and Hodgson, 2011). In the context of Wnt signalling, activation of the β–catenin-independent PCP pathway causes downstream activation of these components and thus drives filopodia extension (Schlessinger et al., 2009). The receptor-tyrosine kinase-like orphan receptor 2 (Ror2) has been identified as a non-canonical co-receptor for Wnts (Oishi et al., 2003). Indeed, binding of Wnt8a to Ror2 has been demonstrated to drive *de novo* biogenesis of filopodia by inducing actin polymerisation via the PCP pathway (Mattes et al., 2018) (Fig. 4). Wnt proteins are thought to regulate their dissemination from producing cells in this way, as both *Ror2* and *Wnt8a* expression correlate with the number of filopodia. Concordantly, expression of the dominant-negative mutant *ror2*$^{3I}$ in zebrafish embryos reduces the number of filopodia;

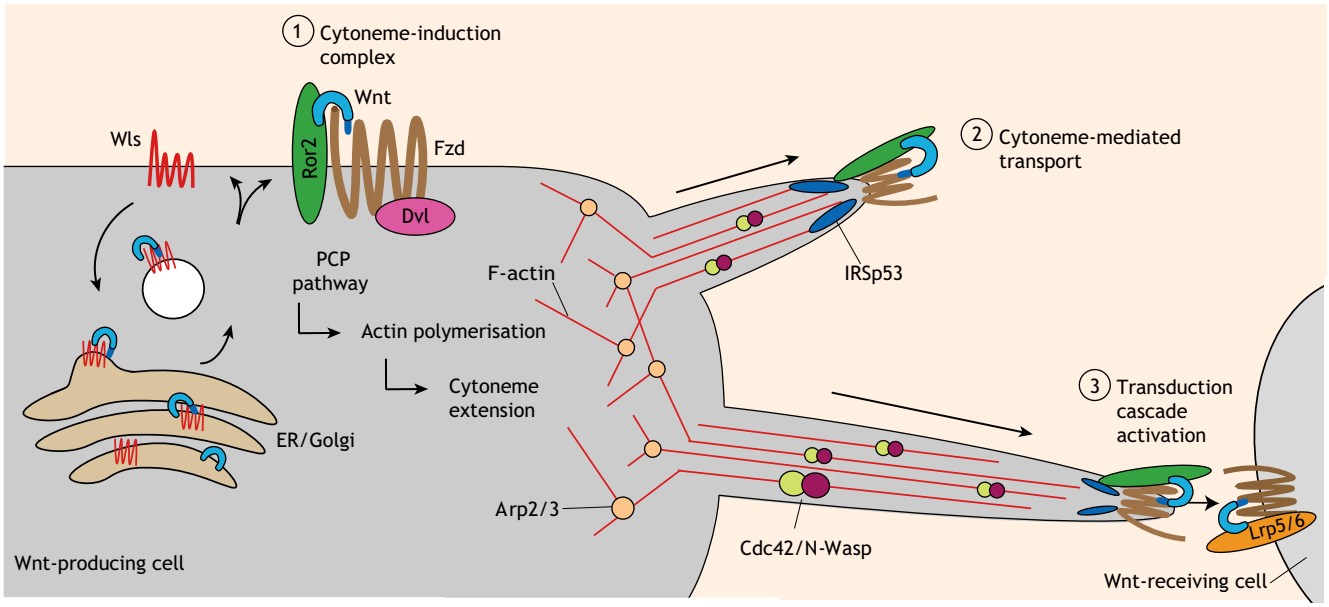


**Fig. 4. Cytoneme-mediated Wnt transport.** Wnt binds to non-canonical Fzd receptors, such as Fzd7, and Ror2, to activate the Wnt/PCP pathway (1). Clustering of Wnt-Fzd receptor complexes causes downstream activation of cytoskeletal regulators, such as Cdc42/N-Wasp, and thus actin polymerisation, which drives the extension of Wnt-bearing cytonemes from the Wnt-producing cell (2). At the Wnt-receiving cell, Wnt binds to Fzd and the co-receptor Lrp5/6 to induce β-catenin-dependent signalling and thus the expression of Wnt target genes (3).

Development (2019) 146, dev176073. doi:10.1242/dev.176073

corresponding with a significant reduction of target gene expression in neighbouring cells and suggesting that Ror2-dependent cytonemes are capable of transporting and delivering Wnt to target cells (Mattes et al., 2018).

Are cytonemes a general mechanism used for Wnt dissemination? A similar Ror2-dependent mechanism for the regulation of Wnt cytonemes was described *in vitro* in gastric cancer (GC) cells, which display upregulated Wnt signalling activity (Chiurillo, 2015; Flanagan et al., 2017; Mattes et al., 2018). Modulation of Wnt cytonemes also influences Wnt-mediated proliferation of GC cells (Mattes et al., 2018). Furthermore, there is emerging evidence that cytonemes are also present in the intestinal crypt in the mucosa of the small intestine in the mouse (Snyder et al., 2015). The intestinal crypt cells need high Wnt activity to regulate the fast cell proliferation that replenishes the intestinal epithelium; these cells migrate up the crypt/villus axis and are shed into the gut lumen. The stromal cells localised around the crypt have been identified as essential Wnt sources (Greicius et al., 2018; Shoshkes-Carmel et al., 2018). Similarly, epithelial Paneth cells in the small intestine and Reg4-positive cells in the colon also secrete Wnts to contribute to the intestinal stem cell niche (Sato et al., 2011; Sasaki et al., 2016). Co-cultivation of intestinal myofibroblasts with *Porcn*$^{-/-}$ crypt cells, which generate Wnt-deficient cells, leads to the induction and maintenance of intestinal crypt organoids. Knockdown of *Ror2* in these myofibroblasts prior to co-culture not only reduced the number of filopodia but also attenuated organoid formation (Mattes et al., 2018). Together, these results highlight a role for cytonemes in transporting Wnt in several vertebrate tissues in order to regulate stem cells and tissue homeostasis.

Cytoneme extension can also be modulated by HSPGs. In *Drosophila*, depletion of the glypicans Dally or Dlp significantly reduces the expansion of cytonemes, and cytonemes are rarely detected in *dally/dlp* double mutants (González-Méndez et al., 2017). In the context of Hh signalling, contacts between cytonemes from anterior and posterior compartment cells are thought to be stabilised by trans interactions between glypicans and Ihog, a co-receptor of Hh; overexpression of either stabilises the cytonemes and contact points (González-Méndez et al., 2017). As Hh is another lipid-modified morphogen, a similar mechanism may be conceivable for cytoneme-mediated delivery of Wnt, although the effects of perturbing HSPG function on Wnt-positive cytonemes has not yet been evaluated.

It has also been suggested that cytonemes act as conduits in a system where exosomes act as the carrier. In *Drosophila*, the localisation of Hh to cytonemes appears to occur in a punctate fashion, with puncta moving along the cytoneme. Owing to their size and the observed colocalisation of Hh and its co-receptor Ihog with the exosomal marker CD63-GFP, these were suspected to be exosomes (Gradilla et al., 2014). Although inhibiting MVB biosynthesis has been shown to reduce Hh secretion and gradient formation, this was not evaluated in the context of Hh localising to cytonemes. It would be interesting to assess perturbations in the localisation of Hh puncta to cytonemes upon inhibition of exosome synthesis, as a reduction could suggest a role for exosomes in transporting Hh on cytonemes. Because Wnt also colocalises with exosomal markers, and Wnt puncta have been detected on cytonemes, this begs the question of whether a similar mechanism is utilised in transporting Wnt (Stanganello et al., 2015). More recently, an interaction between exosomes and filopodia has been reported in the delivery of exosomes to target cells. Here, exosomes are seen to 'surf' along the filopodia before being endocytosed at the filopodia base, which appear to be endocytic hotspots (Heusermann et al., 2016). It may be speculated that morphogen-containing exosomes could also interact with cytonemes at target cells. Although these mechanisms have not been studied in the context of Wnt, interactions between exosomes and cytonemes cannot be ruled out and may offer a viable, synergistic view for Wnt trafficking.

In summary, there are specific mechanisms to disseminate Wnt proteins, which are used in a context- and tissue-specific manner in a variety of organisms (Table 1). Thus far, these potential mechanisms of extracellular Wnt transport have primarily been viewed in a mutually exclusive manner. However, the evidence discussed here suggests that these mechanisms may cooperate in the delivery of Wnt to target cells.

## Activation of signal transduction in the target cell: the 'hand-over problem'

Regardless of the method of extracellular transport, once Wnt has reached the target cell it has one final hurdle to overcome: the handover problem. This describes the issue of how Wnt is transferred from the carrier to the receptor complex on the receiving cell. At the surface of the receiving cell, Wnt binds to its cognate Fzd receptors and co-receptor Lrp5/6, which cluster (along with intracellular binding proteins, such as Dishevelled) to form a large complex of receptors and ligands, termed the Wnt signalosome (Bilić et al., 2007; Gammons et al., 2016). This complex is then endocytosed into the cell, transducing the Wnt signal by inhibiting the formation of the destruction complex, and thus permitting the accumulation of β-catenin and downstream transcription of target genes (Brunt and Scholpp, 2018). A prerequisite for this, however, is the dissociation of Wnt from any bound chaperones or vesicles before it can bind to the Wnt-receiving Fzd receptors.

For the Wnt-binding protein afamin, this hurdle might be overcome by its flexible structure. Superimposing the crystal structures of two independent afamin models, which differ by their crystallographic packing environment, reveals slightly different secondary structures in domains I and III. A key difference is that the conformational change in domains I and III reveals a hydrophobic cleft. A multi-conformer model has therefore been proposed, suggesting that upon ligand binding afamin undergoes a conformational change to accommodate the palmitate moiety of Wnt3a in its hydrophobic binding pocket (Naschberger et al., 2017). This conformational flexibility of afamin permits a model whereby Wnt3a can be released from afamin to allow Wnt to bind to the receiving Fzd receptor. The exchange is likely driven by a higher affinity of Wnt3a for Fzd, although this is yet to be examined (Wilson, 2017).

The varying affinity of Wnt proteins for different receptors can also explain how Wnt can be passed from one receptor to another (i.e. from the Wnt-producing to the Wnt-receiving cell membrane). For example, Wnt8a has been observed to cluster with Ror2 and Wls on the tips of cytonemes, and is then delivered to Fzd-expressing receiving cells (Mattes et al., 2018). This suggests a model whereby Wnt8a is released from Ror2 and binds to Fzd/Lrp6 complexes, which induces signalosome formation and endocytosis. These ligand-receptor complexes have been shown to traffic to late endosomes for recycling or degradation (Hagemann et al., 2014). However, the observation that Ror2 is endocytosed alongside Wnt8a in the receiving cell raises the question of whether the Ror2 complex is recycled via the same route. If so, how is this achieved? Is the cytoneme tip cleaved and endocytosed or could cytoneme-localised Wnt-bearing exosomes be released from the tip and endocytosed into the receiving cell?

The endocytosis of exosomes into recipient cells has previously been reported, including the delivery of Wnt11-positive exosomes in breast cancer cells (Luga et al., 2012). It is also possible that exosomes might fuse with the cell membrane to release its contents

into the cytoplasm. However, exosomes have been observed to be loaded into recycling endosomes in the recipient cells, suggesting endocytic uptake (Théry et al., 2009). As previously mentioned, this could be mediated by HSPGs, which may act as endocytosis receptors (Christianson and Belting, 2014). At what point is Wnt released from the exosome to the receiving cell? Does this occur at the cell surface prior to endocytosis, or is the signal transduced in the cytosol? Furthermore, does this occur as part of a bulk endocytosis event or does Wnt interact with specific cell surface receptors to mediate cell-specific endocytosis?

It is clear from the findings mentioned above that the solution to the final activation of signal transduction remains elusive, and much like the mechanisms for extracellular transport, an open-minded approach is required, as the handover of Wnt carrier to receiving cell may also occur in a context-dependent and cell-specific manner.

## Concluding remarks

By discussing evidence for each of the proposed mechanisms of extracellular Wnt transport (HSPG-aided diffusion, Wnt chaperones, lipoproteins, exosomes and cytonemes), we conclude that although free diffusion is unlikely, the solution to this problem is multi-faceted. The ability to inhibit Wnt signalling through inhibition of each of these mechanisms, in a variety of organisms, highlights the diversity of Wnt transport mechanisms, which are likely utilised in a context- and tissue-specific manner. A flexible view must, therefore, be adopted concerning interactions between these mechanisms, without ruling out the possibility of these mechanisms working in concert with one another.

There remain several unanswered questions, most notably at a molecular level. Insights into the molecular interactions of Wnt with other proteins, particularly at crucial handover events, could provide an insight into how Wnt is passed between structures, from the loading of Wnt onto transport machinery to its handover at the target cell surface, which may require a more biophysical approach to assess binding affinities and dynamics. Continuing from this, how are these processes regulated? Do Wnt proteins regulate their secretory routes based on their PTMs, or is its mechanism of transport pre-determined by the polarity and gene expression of different cell types? To clarify the mechanisms surrounding Wnt transport, the future questions we ask must shift from a matter of ‘where’ Wnt is transported, to ‘how’ Wnt is transported.

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