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COMMENTARY

Role of cytonemes in Wnt transport

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ABSTRACT

Wnt signaling regulates a broad variety of processes during embryonic development and disease. A hallmark of the Wnt signaling pathway is the formation of concentration gradients by Wnt proteins across responsive tissues, which determines cell fate in invertebrates and vertebrates. To fulfill its paracrine function, trafficking of the Wnt morphogen from an origin cell to a recipient cell must be tightly regulated. A variety of models have been proposed to explain the extracellular transport of these lipid-modified signaling proteins in the aqueous extracellular space; however, there is still considerable debate with regard to which mechanisms allow the precise distribution of ligand in order to generate a morphogenetic gradient within growing tissue. Recent evidence suggests that Wnt proteins are distributed along signaling filopodia during vertebrate and invertebrate embryogenesis. Cytoneme-mediated transport has profound impact on our understanding of how Wnt signaling propagates through tissues and allows the formation of a precise ligand distribution in the recipient tissue during embryonic growth. In this Commentary, we review extracellular trafficking mechanisms for Wnt proteins and discuss the growing evidence of cytoneme-based Wnt distribution in development and stem cell biology. We will also discuss their implication for Wnt signaling in the formation of the Wnt morphogenetic gradient during tissue patterning.

KEY WORDS: Cytoneme, Signaling filopodia, Wnt, Extracellular transport

Introduction

During embryogenesis, all multicellular organisms face the same fundamental challenge: the development of a complex structure originating from a single cell. One of the first steps of development is the establishment of the embryonic body plan. Lewis Wolpert formulated a model for the generation of positional information through form-giving substances known as morphogens (Wolpert, 1969). He proposed that these morphogens form concentration gradients within a tissue and that a specific threshold of the morphogen determines cellular identity. Since 1969, the concept of morphogen gradients has received substantial experimental validation (Gurdon and Bourillot, 2001). The first Wolpertian morphogenetic gradient identified was that of bicoid protein, which forms along the anterio-posterior axis of a *Drosophila* embryo in order to determine its longitudinal organization (Driever and Nüsslein-Volhard, 1988). In the following years, similar morphogenetic activities involving hedgehog/sonic hedgehog (Hh/Shh), retinoic acid, transforming growth factor beta (TGFβ) and fibroblast growth factor (FGF) signaling were discovered (reviewed in Bökel and Brand, 2013). The best characterization of wingless/int (Wnt) morphogen activity involves β-catenin-dependent or canonical Wnt signaling (reviewed in Logan and Nusse, 2004). Paracrine signaling activity is fundamental to the morphogenetic function of Wnt proteins in tissue patterning. However, the extracellular transport mechanism of this morphogen from the signal-releasing cell to the recipient cell is still debated. Recent data suggest that Wnt proteins are distributed on long signaling filopodia known as cytonemes, which allow contact-dependent, juxtacrine signaling over a considerable distance. We have recently shown that, in zebrafish, Wnt8a is transported on actin-containing cytonemes to cells, where it activates the signaling required for the specification of neural plate cells (Stanganello et al., 2015). Concomitantly, the Wnt receptor Frz was identified on cytonemes to enable the retrograde transport of Wnt proteins on these protrusions during flight muscle formation in *Drosophila* (Huang and Kornberg, 2015). In chicken, Frz7 was found on extensions that are positive for actin and microtubules during dermomyotome development (Sagar et al., 2015).

In this Commentary, we discuss this distinct molecular mechanism of Wnt trafficking and its implications for the formation of the Wnt morphogenetic field in expanding tissue. We first introduce Wnt function by focusing on paracrine signaling, before discussing the different modes of extracellular Wnt trafficking by highlighting recent evidences of a cytoneme-mediated transport in development and in the stem cell niche. This unexpected trafficking mode raises numerous questions with regard to morphogenetic gradient formation within a growing tissue, which we will address in the concluding section of the article.

Wnt/β-catenin as a morphogenetic signal for tissue patterning

Canonical Wnt proteins are important for tissue patterning, the regulation of cell adhesion and cell proliferation during embryogenesis, tissue homeostasis and regeneration (reviewed in Clevers, 2006; Clevers and Nusse, 2012; Logan and Nusse, 2004). Deregulation of Wnt signaling leads to severe consequences, such as birth defects, neurodegenerative diseases and cancer. Wnt ligands are produced and secreted by a defined subset of cells, then spread throughout a tissue to form a concentration gradient. Wnt proteins have been proposed to act as evolutionary conserved Wolpertian morphogens. Several lines of evidence suggest that the *Drosophila* Wnt protein Wingless acts as a long-range morphogen (Zecca et al., 1996; Neumann and Cohen, 1997). *Drosophila* Wnt-recipient cells respond in a concentration-dependent manner by modulating the expression of target genes (Strigini and Cohen, 2000; Kicheva et al., 2007). Although Wnt proteins can spread over a distance of over 20 cells in the *Drosophila* wing imaginal disc (Gallet et al., 2008), there is also evidence that they act in a concentration-independent manner, e.g. by regulating wing growth (Baena-Lopez et al., 2009).

In vertebrates, one of the best examples of the morphogenetic activity of Wnt/β-catenin signaling is its function during neural development. The vertebrate central nervous system (CNS) originates from the embryonic neural plate and can be subdivided along the longitudinal axis in the forebrain, midbrain, hindbrain and

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spinal cord. Numerous reports highlight the importance of Wnt/β-catenin signaling in regionalizing the CNS primordium along the anterio-posterior axis in vertebrates. One of the first indications that Wnt/β-catenin is participating in tissue patterning was uncovered in overexpression experiments involving Wnt8 in *Xenopus laevis*, and which produced headless embryos including the loss of anterior brain structures (Smith and Harland, 1991). These findings were explained by the re-specification of anterior neural tissue to a posterior fate (Fredieu et al., 1997). A posteriorizing role for Wnt/β-catenin was also found in Wnt8c overexpression studies in mice (Pöpperl et al., 1997). Similarly, inactivation of the Wnt repressors T-cell factor 3 (Kim et al., 2000) and Axin1, resulting in increased Wnt signaling, yield microcephalic zebrafish embryos (Heisenberg et al., 2001). Further studies have shown that Wnt8-mediated Wnt/β-catenin signaling activity is required for brain regionalization in a concentration-dependent manner in *Xenopus* (Kiecker and Niehrs, 2001). In zebrafish, zygotic anterio-posterior patterning is predominantly orchestrated by the Wnt/β-catenin family members Wnt8a and Wnt3a (Erter et al., 2001; Lekven et al., 2001). A graded Wnt/β-catenin signal mediated by Wnt8a induces the expression of the posterior hindbrain marker *gbx1* at the expense of the anterior fore- and midbrain marker *otx2* (Rhinn et al., 2005).

These findings indicate that Wnt8 functions as a morphogen in order to shape tissues by defining cellular fate dependent on certain thresholds – higher Wnt8 levels for posterior fates and lower Wnt8 levels for anterior fates. To function as morphogens, Wnts must be secreted from a local signaling center and, subsequently, be distributed over distances of several cell diameters towards the recipient tissue. Cellular memory of the Wnt/β-catenin activity state and mixing of cells from the Wnt-producing population with the Wnt-receiving population might account for Wnt/β-catenin signal distribution in some developmental contexts (Aulehla et al., 2003; Serralbo and Marcelle, 2014). However, paracrine signaling is the best explanation for the formation of such a gradient, e.g. in the zebrafish neural plate. Here, Wnt-producing cells are of mesodermal origin and do not intermingle with the ectodermal cells of the neural plate during gastrulation (Keller et al., 2008; Woo, 1997). In summary, the function of Wnt/β-catenin signaling within the neural plate correlates with the formation of a morphogenetic field, and differences in Wnt8a concentrations are responsible for brain subdivisions along the anterio-posterior axis (Bang et al., 1999; Dorsky et al., 2003; Green et al., 2014). Regulation of propagation is fundamental to the formation of a Wnt8a morphogenetic gradient. However, how Wnt proteins are distributed to form this gradient and function over tens of micrometers is still unclear.

Advances in understanding of Wnt protein transport

An extensively investigated mode of morphogen transport is free diffusion, in which morphogens are released and diffuse passively in the extracellular space (Müller et al., 2013). Wnt proteins can be transferred between adjacent cells through lateral diffusion, engaging cell surface molecules, such as heparin sulfate proteoglycans (HSPGs) (Fig. 1A). By analyzing the HSPG mutants Dally and Dally-like, researchers hypothesized that glypicans are required to maintain normal levels of extracellular Wnt at tissue surfaces in *Drosophila* (Han et al., 2005; Takei et al., 2004). However, long-range spreading of Wnt proteins by diffusion is unlikely, as these ligands are post-translationally lipidated, which generates a poorly soluble, hydrophobic molecule (Willert et al., 2003; Janda et al., 2012). Wnt proteins might be transported through multi-protein complexes that mask their hydrophobic lipid modifications and increase solubility. These complexes can either be formed by Wnt proteins themselves, forming micelles, or by other lipid-binding proteins that then serve as shuttles. The existence of Wnt-binding proteins that facilitate the diffusion of Wnt proteins has recently been suggested in *Drosophila* and *Xenopus* (Fig. 1B). Wnt has been shown to be bound by secreted wingless-interacting molecule (Swim), which facilitates its spread (Mulligan et al., 2011). Furthermore, the signaling range of Wnt can be increased by extracellular proteins that belong to the soluble frizzled-related protein (sFRP) family (Gorny et al., 2013; Mii and Taira, 2011).

In addition to carrier proteins, exovesicles have been proposed to play a role in the passage of hydrophobic Wnt molecules through tissue (Fig. 1C) (reviewed in Gross and Boutros, 2013). In *Drosophila*, exovesicles are associated with Wnt proteins and move at the same speed as has previously been observed for Wnts (Greco

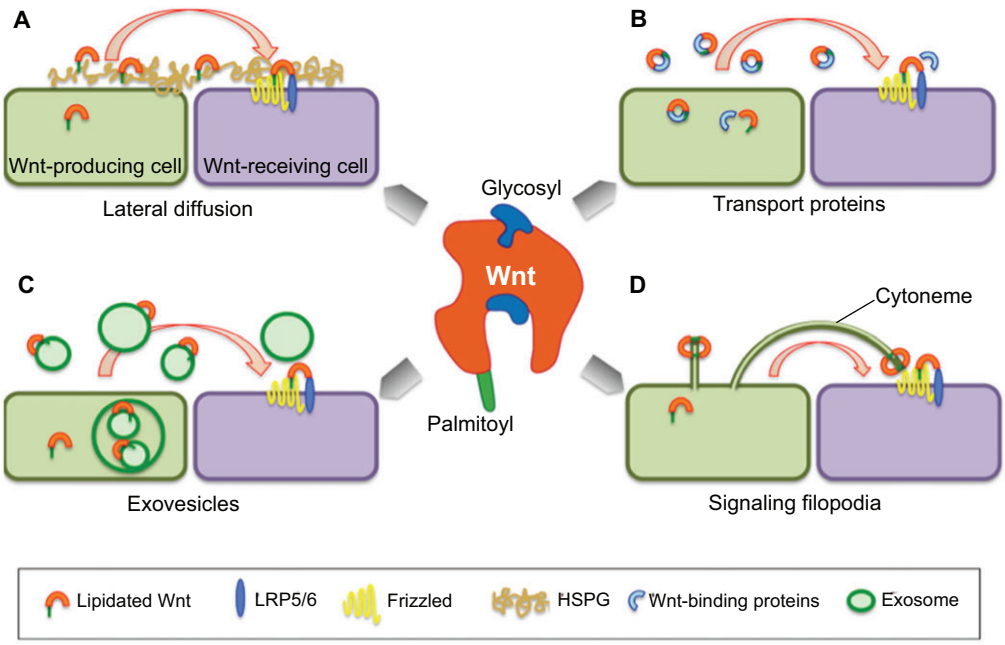

Fig. 1. Schematic representation illustrating known modes of Wnt transport. (A) Lateral diffusion. Heparin sulfate proteoglycans (HSPGs) facilitate lateral diffusion of the glycosylated Wnt ligand. (B) Transport proteins. Palmitoylated Wnt is bound and solubilized by specific, extracellular lipid-binding proteins and transported to the receiving cell. (C) Exovesicles. Exovesicles, such as lipophorins and exosomes shuttle lipid-modified Wnts on their surfaces and deliver them to the receiving cell. (D) Cytonemes. Here, Wnt ligand is transported through cytonemes that extend from the emitting cell to the receiving cell.

et al., 2001). Indeed, an RNA interference (RNAi)-based knockdown of lipophorin led to a decrease in long-range Wnt signaling (Panáková et al., 2005; Willnow et al., 2007). Similarly, mouse Wnt3a associates with high-density lipoprotein particles in the surrounding medium and remains active (Neumann et al., 2009). However, some recent evidence suggests that another type of exovesicles, exosomes, is important to the mobilization of Wnt proteins (Gross and Boutros, 2013). The release of exosomes requires the transport of cargo proteins through an endocytic route. Prior to its secretion, Wnt proteins are endocytosed and routed to multivesicular bodies (MVBs) to be packaged into vesicles for secretion. The ESCRT-0 complex is required for this process, and its blockage is known to lead to impaired Wnt protein secretion (Coombs et al., 2010). In the *Drosophila* neuromuscular junction, Wnt proteins are mobilized on MVB-derived exosomes to signal via the synaptic cleft to increase postsynaptic sensitivity through glutamate receptor clustering (Kerr et al., 2014; Korkut et al., 2009). In the wing imaginal disc Wnt proteins are presented on the surface of vesicles, and these ligand-containing exosomes induce signaling in target cells (Gross et al., 2012). Recent data indicate a function for Wnt exovesicles in cell–cell communication in tumorigenic tissues (Koch et al., 2014; Luga and Wrana, 2013; Menck et al., 2013). Thus, although Wnt proteins can be mobilized on exovesicles in healthy and diseased tissues, it is unclear whether this mode of transport contributes to the formation of a signaling gradient. Studies in *Drosophila* suggest that, even when Wnt proteins are secreted on exosomes, its morphogenetic gradient forms independently of exosomal transport because the knockdown of GTPase Rab11, which inhibits exosome production, does not influence the formation of Wnt gradients (Beckett et al., 2013). Furthermore, the hypothesis that Wnt proteins must be secreted from the membrane of an 'origin cell' to fulfill their morphogenetic activity has recently been challenged in a study by Alexandre and colleagues, who tethered endogenous Wnt ligands to the membrane (Alexandre et al., 2014). Although the transgenic flies produced in this study were infertile and could not fly, membrane-bound Wnt was able to rescue wing patterning in the *Drosophila wingless* mutant, which suggests that paracrine Wnt signaling is dispensable for wing patterning in this transgenic fly. The above studies have initiated a debate with regard to the cellular mechanisms that ensure the controlled release and spread of Wnt proteins from origin cells in order to generate a morphogenetic gradient field in a neighboring tissue.

Cytonemes regulate the distribution of signaling proteins

Another suggested transport mechanism of morphogens is through specialized signaling filopodia. Filopodia are thin actin-based protrusions that extend from cells involved in cell adhesion and migration (Jacquemet et al., 2015). In 1961, Wolpert and Gustafson visualized filopodia in live sea urchin embryos at the blastula stage, resulting in behavior indicating that filopodia function as sensors of patterning information (Gustafson and Wolpert, 1961). In 1999, by using live imaging, Ramírez-Weber and Kornberg detected one class of long and fragile cellular protrusions in *Drosophila* and speculated that they mobilize signaling molecules (Ramírez-Weber and Kornberg, 1999). These cell extensions with signaling functions were defined as cytonemes and have been detected in various animal models in the following years (Kornberg and Roy, 2014). In fact, air sac precursors extend cytonemes towards FGF-producing cells (Sato and Kornberg, 2002). Moreover, in the *Drosophila* wing disc, apical cytonemes are known to orient toward the source of the bone morphogenetic protein decapentaplegic (Dpp) (Hsiung et al., 2005). Notch/Delta signaling promotes the long-range lateral inhibition of pro-neural fates through signaling filopodia in the *Drosophila* notum (Cohen et al., 2010; De Joussineau et al., 2003). In addition, cytonemes that express epidermal growth factor (EGF) receptor have been reported to extend towards a source of EGF in the eye disc (Roy et al., 2011). Cytonemes have also been suggested to be required for the formation of a hedgehog gradient in *Drosophila* epithelial cells (Rojas-Rios et al., 2012; Bischoff et al., 2013). Furthermore, a cytoneme-based transport has been documented in the limb buds of chicken embryos (Sanders et al., 2013). A key feature of cytonemes is their signal molecule specificity. Different signaling proteins are trafficked by different cytonemes, although these signaling filopodia may have similar appearances (Roy et al., 2011). Whether such a modality for the transport and signaling of lipid-modified ligands or receptors of the Wnt signaling pathway is used has been debated within the community until recently.

Wnt cytonemes in development

There is a growing body of evidence indicating that Wnt ligands can be mobilized on cell protrusions. Wnt2b is transported on cellular extension to a Wnt-recipient cell in *Xenopus* fibroblast cell culture (Holzer et al., 2012). In zebrafish, dependent on lipid modification, Wnt8a is localized to the membrane, including to cellular protrusions (Luz et al., 2014). However, until recently, the implications of these findings have not been fully understood. We were able to show that Wnt8a accumulates on the plasma membranes of Wnt-producing cells (Stanganello et al., 2015). These Wnt8a clusters recruit the transducer of Cdc42 -dependent actin assembly protein 1 (Toca-1; also known as fnbp1l), which determines the positions of new filopodia by locally activating the filopodia nucleation complex (Ho et al., 2004). Subsequently, these growing filopodia transport Wnt8a protein at their tips to the target cells. These protrusions can be characterized as actin-containing filopodia, which are necessary and sufficient to distribute Wnt proteins within the neural plate (Fig. 2A,C). We have found activated Cdc42/N-Wasp in forming Wnt-containing filopodia, suggesting that a dynamic actin polymerization takes place during filopodial outgrowth (Stanganello et al., 2015). Wnt8a is then transported as a cluster on the filopodial tip. When the extended Wnt8a-positive filopodia reach their target cells, the Wnt pathway in the plasma membrane of the recipient cells is activated by inducing the Wnt-receptor complex, low-density lipoprotein receptor-related protein 6 (Lrp6) signalosome, at contact points (Stanganello et al., 2015). The 'Lrp6–signalosome' is a dynamic structure that mediates Wnt signaling (Niehrs, 2012). After contact, the filopodia are pruned off and a Wnt8a-positive cluster remains attached to the membrane of the responding cell (Stanganello et al., 2015). Such remains of the Wnt-positive filopodia have been previously observed as exovesicles – i.e. membrane vesicles released by dendritic cells – (Luz et al., 2014), but it is unknown whether they provide a further means to mobilize Wnt. *In vivo* imaging suggested that, after contact has been made, Wnt-containing Lrp6–signalosomes are primarily endocytosed into the recipient cells – a prerequisite for signal activation (Hagemann et al., 2014; Stanganello et al., 2015). On the basis of the definition of cytonemes as signaling filopodia that activate paracrine signaling in a distant cell (Kornberg and Roy, 2014), we, hereafter, refer to these cellular extensions as Wnt cytonemes.

The function of the Wnt-recipient cell in Wnt signaling has also been investigated. Data from *Drosophila* provided the first evidence

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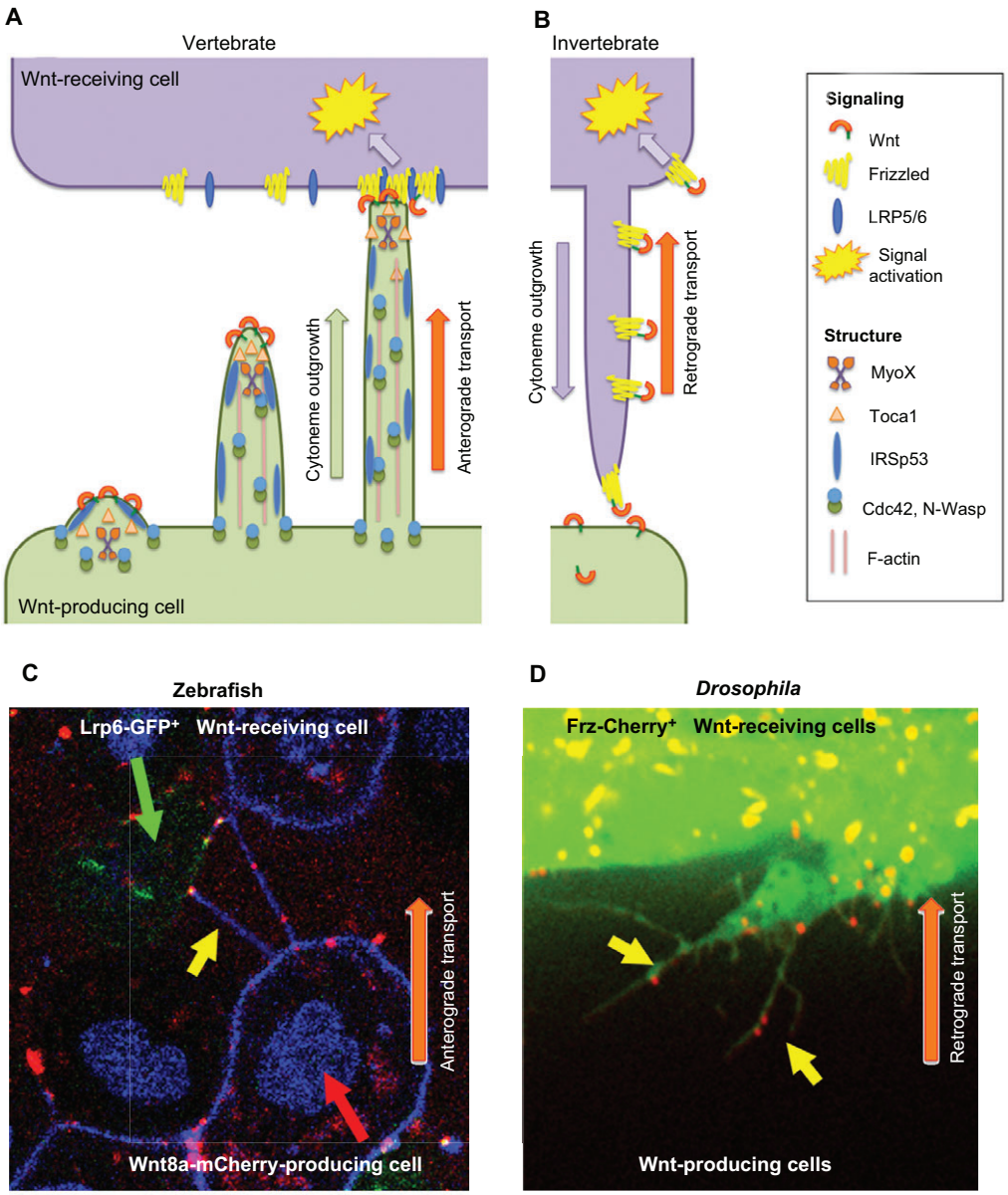

Fig. 2. Structure and function of Wnt cytonemes. (A) Vertebrates. In zebrafish, Wnt8a is recruited into clusters together with the nucleation protein Toca-1 to the plasma membrane. Then, Cdc42 and N-Wasp, together with IRSp53, induce the formation of actin-containing filopodia, the cytonemes. Wnt cytonemes deliver Wnt8a to the recipient tissue, allowing juxtacrine signaling. In the receiving cell, Wnt8a is endocytosed to activate the Wnt signal transduction cascade. (B) Invertebrates. In *Drosophila*, Frz-positive cytonemes extend from the Wnt-receiving cell to contact a Wnt-producing cell. Following formation of the Wnt-receptor complex at the cytoneme, the ligand is then transported to the Wnt-receiving cell in a retrograde manner. (C) Live imaging of Wnt8a–mCherry-producing zebrafish cells marked with a memCFP and nucCFP (red arrow). These Wnt source cells generate cytonemes (yellow arrow) to contact the cell body of the receiving cells marked by Lrp6-GFP (green arrow) during zebrafish gastrulation. (D) Frz-Cherry-expressing myoblasts (marked with CD8-GFP) sending out cytonemes (yellow arrows) to contact Wnt-expressing air sac cells in *Drosophila*. Image courtesy of Thomas Kornberg, UCSF, CA.

that Wnt signaling components might also be distributed on cytonemes that originate from the recipient cell (Fig. 2B,D). For instance, myoblast progenitors of flight muscles have been shown to send out cytonemes that contain the Wnt receptor Frizzled (Frz) and contact the Wnt-producing cells in the wing imaginal disc; the resulting Wnt–Frz complexes then move in a retrograde manner along the dynamic myoblast cytonemes (Huang and Kornberg, 2015). Similarly, the Wnt receptor Frz7 has been identified on the filopodia of dermomyotome cells in chicken (Sagar et al., 2015). Taken together, there is increasing evidence, suggesting that a conserved filopodia-based mechanism contributes to the distribution of both Wnt ligands and their receptors during development of vertebrate and invertebrates.

Different types of cytoneme in vertebrates

Although cytonemes have been described in detail in *Drosophila*, the characterization of these signaling filopodia in vertebrate tissue is still in its infancy; nevertheless, a few common criteria have emerged. Below, we compare, therefore, cytoneme-mediated transport of Wnt morphogens in zebrafish (Stanganello et al., 2015) with the cytoneme-mediated transport of Shh in chicken (Sanders et al., 2013) (Fig. 3).

Wnt8a resides at the tips of elongating filopodia that originate from this Wnt8a-producing cell and contact the soma of the Wnt-responding cell (Stanganello et al., 2015) (Fig. 3A). The signaling range of Wnt8a depends on the average length of the filopodia, and cytonemes are up to 50 µm long. The RhoGTPase Cdc42 regulates the appearance of the actin cytoskeleton within Wnt cytonemes. Wnt8a is loaded and transported onto the tips of filopodia, which deliver the morphogen to responding cells. The velocity of propagation is 0.11 µm/s. This transport mode allows juxtacrine signaling over a distance. After this contact is established, the filopodia are pruned off in less than 10 min. In this case, the amount of morphogen received by the responding cell depends on the rate of filopodia growth, on the morphogen concentration at the filopodia tips, and on the frequency of contact between a Wnt-producing cell

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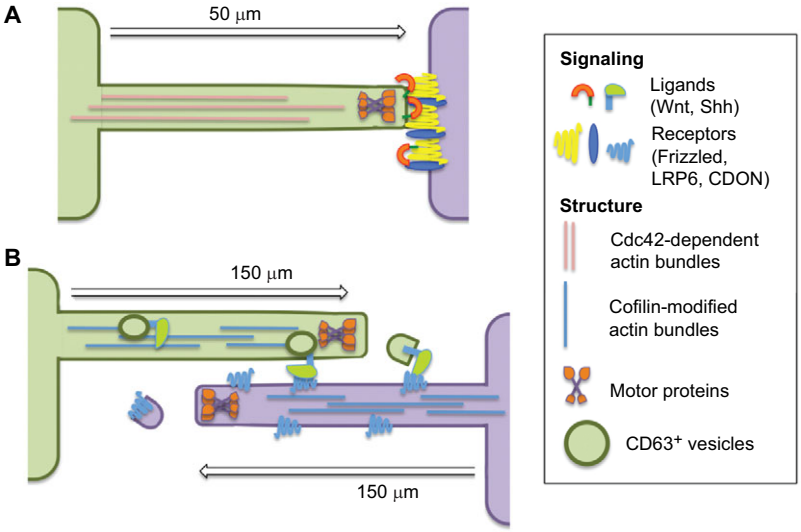

Fig. 3. Comparative analysis of cytonemes transporting Wnts and sonic hedgehog (Shh). (A) Zebrafish. Wnt-containing cytonemes have a length of up to 50 µm and contact the soma of the receiving cell. Wnt is transported at the tip of the cytonemes. (B) Chicken. Shh-containing cytonemes contact CDON-positive cytonemes; in chicken, each of them are up to 150 µm long. Shh is continuously transported along the cytonemes.

and a recipient cell. A morphogen transport mechanism as described for Wnt8a – using short, robust filopodia – might more suitably explain morphogen distribution during gastrulation, which is characterized by highly dynamic tissue rearrangements (Stanganello et al., 2015).

In comparison, the Shh morphogen-producing cell and the responding cell play active roles because the ligand-containing cytonemes contact those with the receptor (Fig. 3B). In the chicken limb bud, cytonemes of Shh-producing cells span several cell diameters and contact cytonemes that are positive for the Shh co-receptors BOC and CDON, which emerge from cells of the responding tissue. Here, the cytonemes carrying the ligand and the co-receptor are 150 µm long, which brings the signaling range to ~300 µm. Although there is no direct evidence for cytoskeletal components, Shh-positive cytonemes contain cofilin and unconventional myosin-X (Myo10), suggesting the presence of actin-positive microfilaments. Shh cytonemes act as cables along which the ligands travel to reach the tips of the Boc-positive cytonemes from recipient cells. Shh is transported in both anterograde and retrograde directions along these filopodia allowing paracrine signaling. Thus, the amount of morphogen that is piped to the recipient cell is highly dependent on the speed at which the ligand is carried along the cytoskeleton network that characterizes the cytoneme. The propagation velocity of the anterograde Shh particles is, with 0.12 µm/s, similar to that of Wnt8a. The speed of ligand propagation is consistent with the activity of the actin-based myosin-motor Myo10 (Berg and Cheney, 2002). Besides propagation velocity, increase of contact time would enhance signal activation in the responding cells. Therefore, cytonemes that are involved in Shh transport operate in a context of slow cell migration and proliferation, such as limb development (Sanders et al., 2013).

The mechanism underlying ligand presentation to the recipient cell remains an open question. Data from *Drosophila* suggest that Shh is transported on CD63-positive vesicles along the cytonemes, which are subsequently shed (Gradilla et al., 2014); presumably, these vesicles originate from MVBs. Similarly, active Wnt proteins can be found on MVB-derived vesicles (Gross, et al., 2012) and, as shown by Korkut et al., such a vesicle-mediated transport mechanism operates in the *Drosophila* neuromuscular junction (Korkut et al., 2009). It is, therefore, tempting to speculate that a vesicle-based, short-range transport is mediating the final step in ligand transport to the recipient cell in invertebrates; however, whether such a mechanism exists in vertebrates is currently unknown.

Thus, the transport mechanisms that are mediated by different cytonemes might differ from another, depending on the tissue involved, the nature of the morphogen and the model organism. It is, therefore, important to analyze any context-dependency on the characteristics of cytonemes and the mechanisms of transport.

Cytonemes in the stem cell niche

On the basis of the work discussed above, it is reasonable to assume that a particular mode of Wnt ligand transport is favored over other mechanisms during specific cellular processes. The stem cell niche may provide such an example. Indeed, the formation of a nanotube network to induce a precise local induction of signaling between stem cells and supporting cells has been shown to regulate stem cell behavior (Inaba et al., 2015). A recent study has revealed the importance of the niche architecture as well as of paracrine Wnt signaling, which regulates cell behavior in a wide variety of cell lineages (Wabik and Jones, 2015). Controlled short-range Wnt signaling from supporting cells maintains stem cell fate and allows the differentiation of more distant cells in the intestinal niche (Sato et al., 2011). In the hair stem cell niche, Wnt signaling has been shown to alter the axis of division of bulge stem cells to generate a new hair follicle (Deschene et al., 2014). All of these examples require a tight control of the signaling range as well as the subcellular induction of Wnt signaling in order to regulate asymmetric stem cell division. Indeed, Wnt3a-coated microbeads have been shown to activate the formation of Lrp6–signalosome foci, which induces polarity and, thus, asymmetric division of stem cell (Habib et al., 2013). Recent data have also provided evidence that the Lgr4- and/or Lrg5-positive stem cells in the intestinal crypt generate long cytonemes to aid the transit of signaling effectors (Snyder et al., 2015). Taken together, cytoneme-mediated transport can explain the focal control of the deposition of signaling proteins to stem cells. However, the delivery mode of Wnt proteins in the stem cell niche remains to be elucidated.

Impact of a cytoneme-mediated transport on the Wnt morphogen gradient

One of the central questions in developmental biology is how secreted morphogens can induce a specific tissue pattern with fixed

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boundaries within a growing tissue. The classic view is that formation of a concentration gradient by diffusion depends on multiple factors, including the molecular nature of the morphogen (e.g. its diffusion coefficient) and its availability at various tissue positions (Kicheva et al., 2012). The Wnt gradient can be shaped and maintained by external factors at different levels. First, it can be influenced in its spread by components of the extracellular membrane, such as HSPGs (Han et al., 2005). Second, the rate of degradation of ligands provides control over their cellular concentration and has been shown to be necessary for the formation of stable signaling gradients. Third, molecules acting as antagonists play a role in regulating the Wnt morphogen signaling range (Glinka et al., 1998; Houart et al., 2002; Wang et al., 1997). However, numeric simulation suggests that all of these mechanisms require several tenths of hours to establish a stable morphogen gradient and to ensure an appropriate tissue pattern. In contrast, the Wnt morphogenetic gradient operates during a few hours of gastrulation, which establishes the anterio-posterior axis of the vertebrate embryo. At this stage, cellular reorganizations characterize the embryo. In addition to the separation of the three germ layers by involution and convergent extension is important for the formation of the embryonic body. These cell migration events have a profound impact on signaling gradients, especially when the latter are based on extracellular diffusion. How does the embryo ensure correct ligand distribution and patterning during such highly dynamic tissue rearrangements?

Direct transport offers a more controlled means of morphogen distribution and, therefore, might be more suitable for the rapid generation of a precise gradient during early development. As discussed above, cytoneme-mediated transport is capable of establishing signal gradients in *Drosophila* and vertebrates (Bischoff et al., 2013; Stanganello et al., 2015), and allows for fast signaling activation and gradient formation in the recipient cell within minutes. Indeed, Wnt cytonemes have been shown to target the receiving cell in ∼7 min (Stanganello et al., 2015). Signal activation also depends on the frequency of contact with the recipient cells. Cells that are located closer to the ligand source are contacted more often by Wnt cytonemes than cells further away. The amount of ligand present might also affect signal activation; however, data that substantiate this assumption are sparse. In addition to signal activation, the resulting morphogenetic Wnt gradient also depends on cytoneme length, the time during which the recipient cells are in reach of the cytonemes, and the stability of the induced ligand–receptor complex (Hagemann et al., 2014; Stanganello et al., 2015). Altering the activity of Cdc42, the key regulator of Wnt cytonemes, might affect cytoneme length and, thus, signaling in the responding tissue, with possibly drastic effects on gradient formation and CNS patterning (Stanganello et al., 2015). An increase in cytoneme length leads to a severe anterior shift of the midbrain-hindbrain boundary and causes a reduction of the forebrain that is comparable to that observed when Wnt proteins are ubiquitously overexpressed. IRSp53 regulates Cdc42 activity exclusively during the formation of filopodia (Kast et al., 2014). Concordantly, overexpression of the mutated IRSp53^{4K} blocked Cdc42 activity, resulting in shortening Wnt-positive cytonemes. Indeed, shorter Wnt cytonemes lead to an expansion of the forebrain territory, as seen in embryos with reduced Wnt signaling activity. Consistently, this phenotype can be rescued by simultaneously increasing Wnt activity. These examples suggest that cytoneme-based transport is, indeed, able to establish a morphogenetic Wnt gradient of high precision within a short period of time. This is a prerequisite to the accurate patterning of the anterio-posterior axis in a highly motile cell population, such as that found in an embryo during gastrulation. However, methods to interfere specifically with cytoneme-based ligand transport are sparse. So far, the methods used to interfere with cytoneme formation, such as use of actin polymerization inhibitors or activation of Cdc42 function have a variety of additional effects. One of the best tools to interfere with actin-based filopodia is the mutated form of the Cdc42 effector IRSp53^{4K} to block filopodia formation – including cytoneme formation. Therefore, the challenge for the future will be to develop better methods to specifically control the formation of signaling filopodia.

Conclusions and perspectives

Recent studies have begun to characterize a so far unknown transport mechanism for Wnt proteins. This transport relies on cytonemes and allows the precise and controlled transport of the Wnt ligand and, in some cases, its receptor, in a highly migratory pool of cells in invertebrates and vertebrates. The controlled generation of a Wnt gradient by cytonemes is a prerequisite to establishing a morphogenetic Wnt field that allows precise tissue patterning, such as in the vertebrate neural plate. On the basis of these initial findings, the next step is to substantiate our understanding of the molecular mechanisms that control the formation of these signaling filopodia. To date, a rigorous way to specifically regulate cytoneme function has not been achieved. In addition, it is crucial to determine whether other ligands in the Wnt pathway use a similar distribution mechanism. Furthermore, it is important to investigate whether other delivery mechanisms, in addition to those relying on cytonemes, are used in parallel or whether there is a strict tissue-dependency for the specific transport mechanism employed. Considering the widespread functions of the Wnt signaling pathway in multicellular organisms, further research is needed before extracellular Wnt trafficking, its impact on morphogenetic gradient formation and the effect on tissue patterning are fully understood.

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Competing interests

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