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PRIMER

Exosomes in developmental signalling

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ABSTRACT

In order to achieve coordinated growth and patterning during development, cells must communicate with one another, sending and receiving signals that regulate their activities. Such developmental signals can be soluble, bound to the extracellular matrix, or tethered to the surface of adjacent cells. Cells can also signal by releasing exosomes – extracellular vesicles containing bioactive molecules such as RNA, DNA and enzymes. Recent work has suggested that exosomes can also carry signalling proteins, including ligands of the Notch receptor and secreted proteins of the Hedgehog and WNT families. Here, we describe the various types of exosomes and their biogenesis. We then survey the experimental strategies used so far to interfere with exosome formation and critically assess the role of exosomes in developmental signalling.

KEY WORDS: Exosomes, Hedgehog, Signalling, WNT

Introduction

Much of the cell-to-cell communication that occurs during development is mediated by classic ligand-receptor interactions. In many cases, the ligand is a secreted protein. Examples include members of the WNT, Hedgehog (HH) and bone morphogenetic protein (BMP) families. Ligands can also be transmembrane proteins, as is the case for Notch ligands. Although secreted ligands can reach target cells by diffusion, there is a growing realisation that extracellular vesicles (EVs) may contribute to the release and spread of these canonical ligands and/or to mediate the transfer of a variety of biologically active molecules from one cell to another. Such vesicles, packaged with proteins, nucleic acids and lipids, are indeed found in various extracellular environments. They range between 30 and 1000 nm in diameter, and are classified according to their origin, physical properties and function (Gould and Raposo, 2013). For example, EVs formed by the outward budding of the plasma membrane are known as microvesicles or ectosomes (Box 1), whereas EVs originating from the endosomal system are termed exosomes (Cocucci and Meldolesi, 2015). Here, we focus on exosomes and their formation and function in development.

The term exosome was first used to describe small endosome-derived vesicles secreted by reticulocytes as they mature into erythrocytes (Harding et al., 1984; Pan et al., 1985). This was seen as a mechanism for jettisoning proteins no longer required in the mature erythrocyte (Harding et al., 1984; Pan et al., 1985). Exosomes have subsequently been shown to be secreted from many other cell types and have been suggested to contribute to a variety of physiological processes (Box 2), such as stimulatory and tolerogenic responses in the immune system (Chaput and Thery, 2011), tumour progression and metastasis (Costa-Silva et al., 2015; Hoshino et al., 2015; Luga et al., 2012; Muralidharan-Chari et al., 2010), tissue regeneration (Sabin and Kikyo, 2014), and the exchange and spread of pathogenic proteins and organisms (Schorey et al., 2015).

A variety of biomolecules are found inside or at the surface of exosomes. Internal components include HSC70, HSP90 and 14-3-3-ε (also known as YWHAE) (Buschow et al., 2010; Thery et al., 2001), DNA, mRNAs and miRNAs. For a detailed list of the cargo sorted into exosomes, see Vesiclepedia (http://www.microvesicles.org/), a manually curated compendium of molecules (lipids, RNAs and proteins) identified in different classes of EVs by more than 300 independent studies (Kalra et al., 2012). It is thought that these macromolecules could be delivered to the cytoplasm of recipient cells either by direct fusion with the plasma membrane (Montecalvo et al., 2012) or by internalisation followed by back fusion with the limiting membrane of the endosome (Bissig and Gruenberg, 2014; Tian et al., 2014). In addition to carrying soluble cargo, exosomes are also decorated by transmembrane or membrane-associated secreted proteins on their surface. Thus, intact exosomes coming into contact with recipient cells could allow these exposed signalling molecules to engage with their receptors.

Recent work suggests that membrane-associated developmental signals are among those proteins found on the surface of exosomes. Therefore, exosomes could be important mediators of developmental signalling. In order to assess the underlying evidence and to look forward to future research, it is important to understand the cell

Box 1. Microvesicles: a distinct class of extracellular vesicles

Exosomes form from the endosomal system, but extracellular vesicles can also form by outward budding from the plasma membranes. Such vesicles, which tend to be larger than exosomes, are usually termed microvesicles or ectosomes. Microvesicles have the same topology as exosomes and, like exosomes, contain cargo of cytoplasmic origin. The similarity between the two classes of vesicles also extends to the lipid composition of the domains from which they bud (Antonucci et al., 2012; Bianco et al., 2009), and there is often an overlap between the cargoes they contain (Cocucci and Meldolesi, 2015). However, instead of being sorted to microvesicles by ubiquitylation, cargo is directly or indirectly targeted to microvesicles by post-translational addition of lipids, such as myristoylation and palmitoylation, or by higher-order clustering (Shen et al., 2011; Yang and Gould, 2013). Given that microvesicle formation, like exosome formation, involves membrane bending away from the cytosol, it is perhaps unsurprising that various ESCRT proteins have also been implicated in microvesicle formation (Nabhan et al., 2012). Microvesicles are often mistaken for exosomes and vice versa due to their morphological similarities and the fact that both contain ESCRT proteins. However, the cargoes they contain, their origin and the mechanisms governing their formation and release are distinct.

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Box 2. The role of exosomes in physiology and disease

Exosomes have been implicated in a wide range of physiological and pathogenic processes. Perhaps best characterised is the role of exosomes in immune system regulation. Exosomes derived from antigen-presenting cells carry molecules that activate T cells, thus boosting the immunogenic response (Robbins and Morelli, 2014; Thery et al., 2002). Many pathogenic bacteria and viruses hijack the endocytic system for cellular entry and replication; as a result, exosomes released from infected cells often display antigens from these pathogens. Exosomes could also have a deleterious impact in this context since they may aid the spread of pathogen to uninfected cells (Schorey et al., 2015). More recently, the role of exosomes in cancer biology has received much attention (Rak, 2015; Thery, 2015). Although exosomes shed from cancerous cells can help combat tumour growth by expressing tumour antigens (Clayton and Mason, 2009; Iero et al., 2008), they can also promote tumour growth by reprogramming surrounding cells via the transfer of nucleic acids and proteins that activate specific signalling pathways. Exosomes have also been shown to prepare host organs for metastasis by remodelling the extracellular matrix and promoting angiogenesis (Costa-Silva et al., 2015; Hoshino et al., 2015; Luga et al., 2012; Zhang et al., 2015). The composition of cancer cell-derived exosomes is often distinct from that of exosomes from healthy tissue. As such, exosomes obtained from patient serum have great potential as a non-invasive diagnostic screening tool for detection of early-stage cancer (Melo et al., 2015). Finally, exosomes have been implicated in nervous system development and homeostasis, mediating communication between oligodendrocytes and neurons by providing a mechanism for the transfer of protective proteins, mRNA and miRNA (Fruhbeis et al., 2013). As with cancer, exosomes can be hijacked to promote disease in the nervous system. Indeed, plaque-forming proteins that have been linked to many neurodegenerative diseases (Soto, 2003), such as Parkinson's, Alzheimer's and amyotrophic lateral sclerosis, can be transferred from diseased to healthy cells on exosomes (Coleman and Hill, 2015; Emmanouilidou et al., 2010; Feiler et al., 2015; Rajendran et al., 2006; Silverman et al., 2016).

biology of exosome formation. Here, we first review the processes that govern the formation and composition of exosomes. We then critically describe the available evidence for exosome-mediated transfer of developmental signals such as WNTs and HH proteins.

Multiple mechanisms can generate exosomes

Ubiquitylation-dependent targeting to exosomes

Exosomes originate from an endocytic compartment (Fig. 1). Following internalisation, endosomes move inwards and fuse with one another, leading to the formation of early endosomes (Mills et al., 1998). Cargo contained in early endosomes can then be either recycled back to the plasma membrane or degraded in lysosomes. The best-characterised route to lysosomes utilises the endosomal sorting complex required for transport (ESCRT) pathway (Raiborg and Stenmark, 2009), which recognises cargo earmarked for degradation by ubiquitylation (Piper and Luzio, 2007). In addition to recognising and clustering ubiquitylated cargo, the ESCRT complex drives invaginations in the endosomal membrane, where the ubiquitylated cargo is sorted (Hurley and Hanson, 2010). Such inward budding leads to the formation of cargo-containing intraluminal vesicles (ILVs) within these endosomes, which become known as late endosomes or multivesicular bodies (MVBs). The subsequent fusion of MVBs with lysosomes releases the ILVs into the acidic lysosomal environment and results in destruction of their associated cargo (Luzio et al., 2003). However, fusion with lysosomes is not the only fate of MVBs: MVBs can also fuse with the plasma membrane and release ILVs into the extracellular space – these ILVs are termed exosomes (Harding et al., 1983; Johnstone et al., 1987; Pan et al., 1985). Although the mechanisms that determine the fate of MVBs remain to be elucidated, two different populations of MVBs have been reported to co-exist: cholesterol-rich MVBs that are targeted for secretion, and cholesterol-poor MVBs that are destined for degradation (Mobius et al., 2002). This suggests that some recognition event mediates the fate of MVBs. Although interesting, this idea would also require a mechanism that segregates different cargoes in the two populations of MVBs. Furthermore, the limiting membrane of secreted and degraded MVBs must contain different proteins to ensure recruitment of the correct motor proteins and fusion machinery. Thus, although little is known about how distinct populations of MVBs could be generated and appropriately targeted, it is clear that ESCRT components are involved in the formation of at least a subset of exosomes.

There are four distinct ESCRT complexes, termed ESCRT-0, -I, -II and -III, each orchestrating different steps in cargo selection and exosome formation (Henne et al., 2011; Hurley, 2008, 2010). ESCRT-0 is composed of a heterodimer made up of HRS and STAM1/2, both of which recognise ubiquitylated cargoes (Bilodeau et al., 2002; Fisher et al., 2003; Hirano et al., 2006; Ren and Hurley, 2010). HRS, a cytoplasmic protein, is recruited to endosomes via its FYVE domain (Misra and Hurley, 1999; Raiborg et al., 2001b), which binds to phosphatidylinositol 3-phosphate (PtdIns3P), a lipid that is abundant on the surface of pre-MVB endosomes (Stahelin et al., 2002). HRS then recruits clathrin (Raiborg et al., 2001a) thus helping to cluster and corral ubiquitylated cargo (Raiborg et al., 2002, 2006). ESCRT-I and ESCRT-II, which also contain ubiquitin-interaction domains, cooperate with ESCRT-0 to generate a sorting domain with high avidity for ubiquitylated cargo at the site of ILV formation (Hurley, 2010; Raiborg and Stenmark, 2009). At the same time, they recruit ESCRT-III (Babst et al., 2002), which drives membrane deformation and constricts the neck of the resultant invagination (Babst et al., 2002; Chiaruttini et al., 2015; Henne et al., 2013; Tang et al., 2015; Wollert et al., 2009). De-ubiquitylating enzymes (DUBs) then remove ubiquitin from cargo (Agromayor and Martin-Serrano, 2006) and the ATPase VPS4, along with its co-factor VTA1, disassemble the ESCRT complex (Hurley, 2010), allowing its subunits to be recycled. In principle, the inhibition of any of these components is expected to interfere with the formation of ESCRT-dependent exosomes, albeit not without affecting other processes such as lysosomal targeting.

Ubiquitylation-independent targeting to exosomes

Not all cargoes need to be ubiquitylated for MVB/exosome targeting. The ESCRT-interacting protein ALIX (PDCD6IP) has recently been shown to sort the G protein-coupled receptor PAR1 into MVBs in an ubiquitin-independent, but ESCRT-III-dependent manner (Dores et al., 2012). ALIX binds directly to PAR1 and the ESCRT-III component CHMP4 (Dores et al., 2012; Fisher et al., 2007), thus bridging cargo (PAR1) to MVBs (ESCRT-III) independently of ubiquitylation. ALIX can also serve as an indirect adaptor to bring syndecans (transmembrane heparan sulphate proteoglycans) into MVBs in a ubiquitylation-independent manner. Here, ALIX binds syntenin (SDCDP), which, via its PDZ domains, recruits PDZ ligand cargo such as syndecans and CD63 for incorporation into MVBs (Baietti et al., 2012; Grootjans et al., 1997; Latysheva et al., 2006). These examples show that cargoes can be targeted to MVBs, and hence exosomes, without the need for ubiquitylation and recognition by ESCRT-0, -I and -II. Therefore, manipulating ESCRT-III may be the best way to perturb the formation of all ESCRT-dependent

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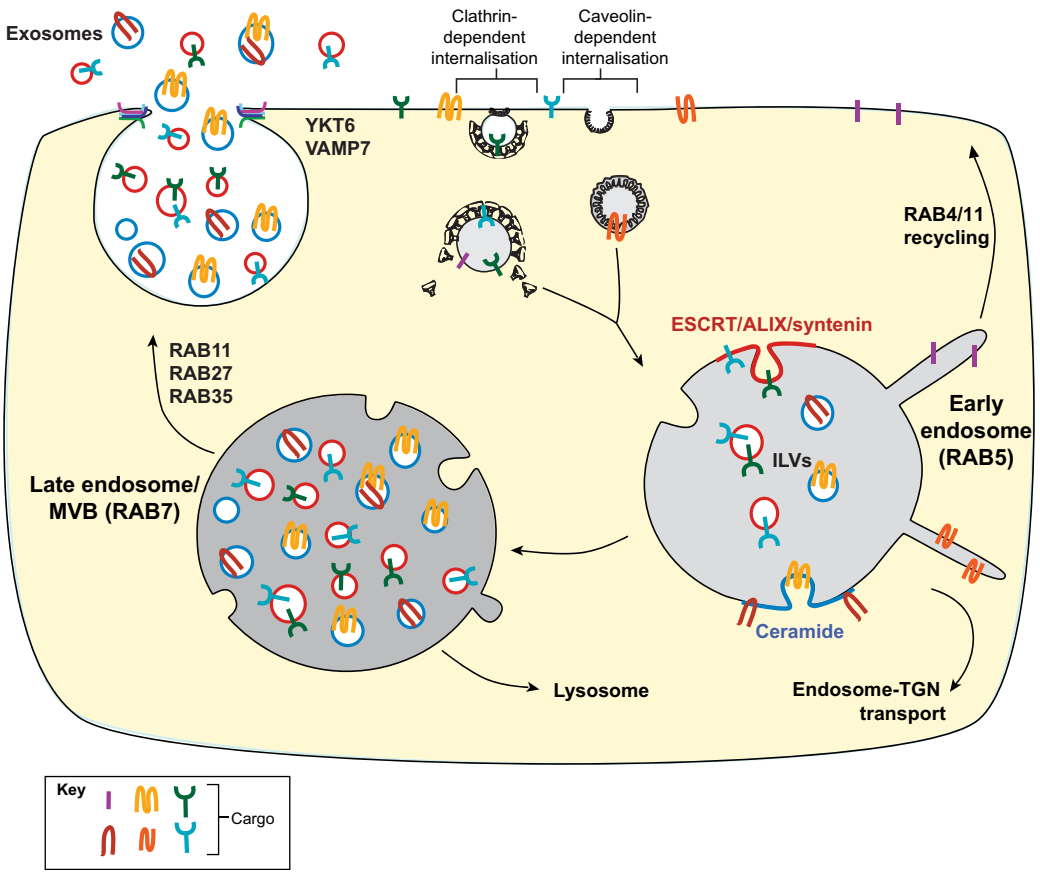

Fig. 1. Exosome biogenesis. Exosomes originate from an endocytic compartment. Following internalisation, endosomes move inwards and fuse with one another to form RAB5-positive early endosomes. Inward budding of the endosomal membrane, driven by the ESCRT complex or ceramide-rich domains, then leads to the formation of cargo-containing intraluminal vesicles (ILVs) within these endosomes, which become known as late endosomes or multivesicular bodies (MVBs). Cargo contained in these endosomes can then be either targeted to lysosomes or recycled back to the plasma membrane (through the activity of RAB11, 27 or 35), where fusion with the membrane is mediated by SNARE proteins such as YKT6 and VAMP7 and results in the release of exosomes. TGN, trans-Golgi network.

exosomes. However, this is unlikely to abolish exosome formation completely.

The formation of ESCRT-independent exosomes

MVBs are still observed following depletion of the four ESCRT subunits (Stuffers et al., 2009), suggesting that exosomes can form independently of ESCRT (Fig. 2). Indeed, the exosomal protein PLP (proteolipid protein) is sorted into MVBs from a lipid-enriched endosomal subdomain devoid of clathrin, independently of ESCRT (Trajkovic et al., 2008). Mass spectrometry analysis revealed that these PLP-containing exosomes are enriched in cholesterol, ceramide and sphingomyelin (SM), a composition that is remarkably similar to that of the endosomal lipid rafts that PLP localises to (Brugger et al., 2006; Ogawa et al., 2011; Trajkovic et al., 2008; Wubbolts et al., 2003). Moreover, perturbation of the neutral sphingomyelinase (SMase; also known as SMPD2), which converts sphingomyelin into ceramide, by either RNAi- or small molecule-mediated inhibition, reduces exosome release, while the addition of exogenous SMase or C_6-ceramide increases exosome release (Trajkovic et al., 2008). These data lead to the hypothesis that ceramide-rich lipid patches on endosomes can induce inward budding and MVB formation. These ceramide-enriched ILVs could then be released as exosomes upon MVB fusion with the plasma membrane.

Another possible ESCRT-independent mechanism of exosome biogenesis involves the tetraspanin CD63, a protein often used as a marker of exosomes. CD63 is involved in the formation of ILVs in melanosomes (a lysosome-related organelle) in an ESCRT- and ceramide-independent manner (van Niel et al., 2011). Furthermore, CD63 is required for the formation of MVBs that are morphologically distinct from those formed by ESCRT in HeLa cells (Edgar et al., 2014). By extension, CD63 could drive exosome formation, although one cannot exclude the possibility that CD63 could be a mere passenger of multiple exosome types.

From MVBs to the plasma membrane

For ILVs to be released, MVBs must be transported to the plasma membrane for fusion, a process that is likely to involve a wide array of proteins, including coat proteins, tethers, Rabs and SNAREs, working together to mediate the transportation, tethering and fusion of MVBs with the plasma membrane (Cai et al., 2007). Identifying specific mediators of these steps is important, as their manipulation could open the door to assessing the role of exosomes in various processes. An RNAi screen has implicated several Rabs and associated proteins in the trafficking of mature MVBs (Ostrowski et al., 2010). For example, it was found that RAB27 knockdown prevents the docking of MVBs to the plasma membrane without affecting MVB content or morphology. As expected, two RAB27 effector proteins, SLP4 (also known as SYTL4) and SLAC2B (EXPH5), were also found to affect exosome secretion. However, unlike RAB27, these effectors appeared to function also in the classical secretory pathway (Ostrowski et al., 2010). Additional Rabs – RAB2B, RAB9A and RAB5A – were further identified as

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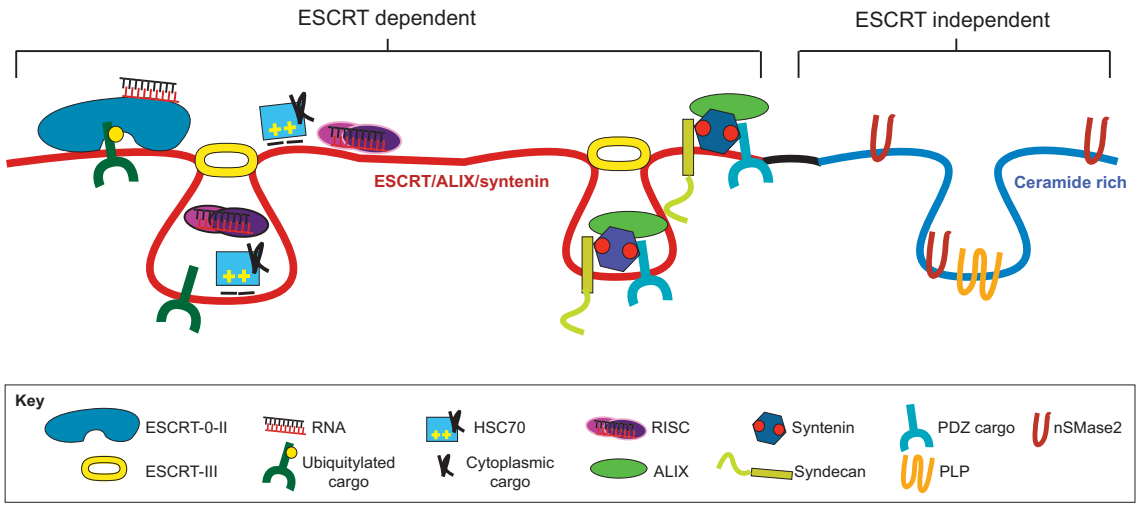

Fig. 2. The sorting of internalised cargo into exosomes. Upon reaching the endosome, internalised cargo is sorted into exosomes/intraluminal vesicles (ILVs) within the lumen of endosomes. Here, the limiting membrane of early endosomes is shown, with its lumen at the bottom and the cytoplasm at the top. There are primarily two distinct endosomal-sorting domains that give rise to exosomes: a clathrin-enriched ESCRT domain (red) and a ceramide-enriched domain (blue). Various processes and components are involved in recognising cargo and generating ILVs at these domains, including those involved in the proposed targeting of cytosolic cargo and RNA to exosomes via interactions with HSC70 and the RNA-induced silencing complex (RISC) (Geminard et al., 2004; Gibbings et al., 2009). After maturation, ILV-containing endosomes can fuse either with lysosomes to degrade the ILVs or with the plasma membrane to release the ILVs as exosomes. nSMase 2, (SMPD2).

mediators of exosome secretion; however, given the importance of these proteins in various endosomal sorting pathways, their perturbation is likely to have pleotropic effects. Another protein that seems to contribute to exosome function is the Rab-GAP, TBC1D10 (Hsu et al., 2010). This protein stimulates the GTPase activity of RAB27 (Itoh and Fukuda, 2006), once again linking RAB27 to exosome secretion. TBC1D10 additionally activates RAB35, which has also been shown to contribute to exosome secretion (Hsu et al., 2010). Yet another Rab family member implicated in exosome production is RAB11 (Beckett et al., 2013; Koles et al., 2012; Savina et al., 2002). Importantly, the suppression of any one individual Rab family member is not sufficient to completely block exosome secretion, perhaps because of redundancy. Alternatively, it is possible that each of these Rabs could govern the secretion of a subset of exosomes.

SNARE proteins mediate the fusion of vesicles with their target membrane compartment and are therefore likely to contribute to the release of ILVs as exosomes. Vesicle SNARES (vSNAREs) located on the MVB recognise target SNARES (tSNAREs) located at the plasma membrane to drive fusion of the two membranes (Cai et al., 2007). The exact machinery required for MVB plasma membrane fusion has not been fully elucidated. The SNARE protein VAMP7 has been suggested to mediate exosome secretion, although this has been disputed (Fader et al., 2009; Proux-Gillardeaux et al., 2007). The *Drosophila* SNAREs YKT6 and Syntaxin 1A have been suggested to regulate exosome-mediated secretion of Wingless (Gross et al., 2012; Koles et al., 2012). However, this suggestion is difficult to evaluate because these SNARE proteins contribute to fusion at many sites in the secretory and endocytic pathways (Jahn and Scheller, 2006). The upshot is that so far no SNARE protein is known to be specifically involved in exosome release.

The role of exosomes in developmental signalling

Given their varied cargoes, and their role in the transfer of biomolecules (see Box 1), exosomes have been investigated as potential mediators of signalling during development, particularly in the context of WNT and HH signalling. The WNT and HH signalling pathways regulate growth and patterning during embryonic development (Perrimon, 1994; Perrimon et al., 2012; van Amerongen and Nusse, 2009), as well as stem cell numbers in epithelia such as those of the skin and intestine (Clevers et al., 2014). Unlike most extracellular signalling proteins, HHs and WNTs are modified by the addition of a lipid during their biosynthesis. This raises interesting issues because the lipid is likely to affect the trafficking, solubility and diffusion of these signalling proteins in the extracellular space. The requirement of long-range signalling by WNTs and HH has been the subject of debate (Alexandre et al., 2014; Farin et al., 2016; Serralbo and Marcelle, 2014). Nevertheless, both proteins have been shown to act over several cell diameters (Niehrs, 2010; Perrimon et al., 2012; Strigini and Cohen, 1997; Zecca et al., 1996). How lipid-modified, and hence poorly soluble, proteins can achieve long-range action is an important question in cell and developmental biology. One possibility is that WNT and HH may not need to diffuse in order to act at a long range. Indeed, it has been suggested that these proteins could remain tethered to specialised signalling filopodia called cytonemes, which extend to distant cells (Bischoff et al., 2013; Hsiung et al., 2005; Ramirez-Weber and Kornberg, 1999; Stanganello and Scholpp, 2016). However, there is as yet no specific means of preventing cytoneme formation, precluding a direct test of this model. One alternative to cytonemes is that lipoprotein particles, such as those involved in lipid transport in the circulation, might act as vehicles for WNTs and HHs. Specifically, the lipid adduct would insert into the particle's outer lipid monolayer that surrounds the lipid core (Panakova et al., 2005). Another model is that the lipid adduct of WNT or HH could be shielded by an extracellular lipid-binding chaperone, such as SWIM (Secreted Wingless Interacting Molecule), a member of the lipocalin family (Mulligan et al., 2012). Yet another possibility, suggested for HH, is that the lipid adduct could be shielded inside micelles (Goetz et al., 2006; Zeng et al., 2001), a model that is

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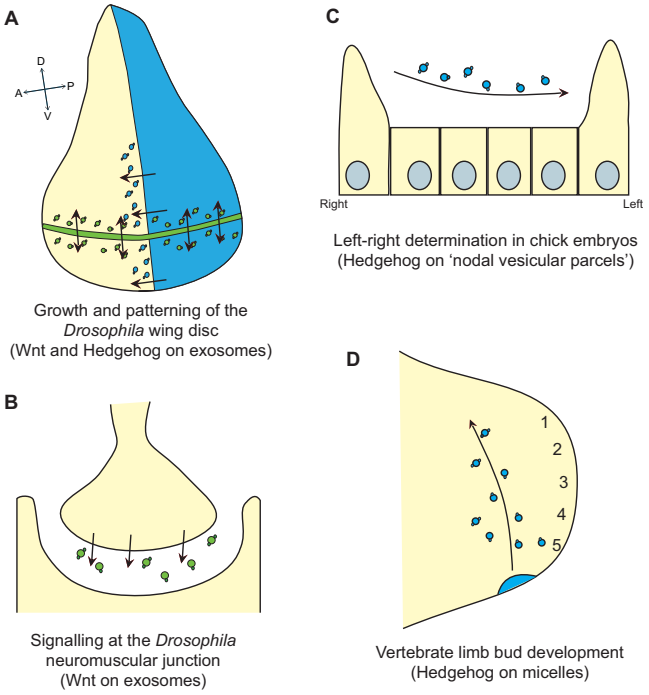

Fig. 3. Putative roles for exosomes in development. Developmental processes in which exosomes have been implicated are depicted. (A) Exosomes could regulate growth and patterning of *Drosophila* wing imaginal discs by facilitating the spread of Wingless (green) from the dorsal ventral boundary and HH (blue) from the posterior compartment. (B) Exosomes could transfer Wingless across the *Drosophila* neuromuscular junction, thus controlling its morphogenesis. (C) Exosome-like structures termed 'nodal vesicular parcels' (NVPs) have been suggested to mediate the spread of HH along the mouse node to reinforce left-right axis specification. (D) HH diffusion controls digit positioning (numbers) in the developing vertebrate limb bud. Here, long-range diffusion is proposed to be mediated by self-aggregating micelles.

difficult to test genetically because it does not invoke the involvement of another protein. Finally, it has been suggested that WNT and HH could be loaded onto exosomes, with the lipid shielded either in the exosome bilayer or by an exosome-associated protein (Gradilla et al., 2014; Gross et al., 2012; Korkut et al., 2009; Matusek et al., 2014; Parchure et al., 2015; Vyas et al., 2014). Below, we focus on the evidence that exosomes could mediate the spread of WNT and HH in developing tissues (Fig. 3).

Exosomes in the context of WNT signalling

The first evidence that WNTs might be released on membrane-bound structures came from studies of Wingless, the main WNT expressed in *Drosophila*. In the *Drosophila* wing imaginal disc epithelium, Wingless was observed in the extracellular space on membrane-associated vesicles termed 'argosomes'. The membranous nature of these vesicles was suggested from the observation that they incorporated GFP appended with a glycophosphatidylinositol (GPI) moiety (Greco et al., 2001). Argosomes were initially thought to originate from MVBs, implying they could be exosomes. However, later work suggested that argosomes constitute lipoprotein particles surrounded by a single layer of phospholipids and the lipoprotein lipophorin (Panakova et al., 2005). Because a human CD63-GFP fusion protein (used here as a marker of exosomes) was found not to colocalise with Wingless in signal receiving cells, it was concluded that lipophorin particles, and not exosomes, are likely to be the main carrier of Wingless along the imaginal disc epithelium. However, it should be noted that CD63-GFP was used as a marker of exosomes because endogenous CD63 is found on exosome preparations from a variety mammalian tissues, but this protein is not endogenous to *Drosophila* and its overexpression could increase membrane fusion and/or alter endosomal morphology (Pols and Klumperman, 2009). Moreover, because CD63 transits through the plasma membrane, it can also mark microvesicles, which bud directly from the cell surface, suggesting that it cannot be used as a specific marker of exosomes. Despite these caveats, CD63 remains a convenient means of marking exosomes both in vertebrate and invertebrate tissues.

Subsequent work, on the role of Wingless at neuromuscular junction (NMJ), revived the interest in exosome-mediated Wingless transport. This was triggered by the observation that Wntless (WLS; also known as Evenness Interrupted and Sprinter), a multipass transmembrane protein that escorts WNTs from the Golgi apparatus to the plasma membrane (Bänziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006), is released from pre-synaptic termini. Such release was shown to be required for the transfer of Wingless across the synapse and for postsynaptic activation of Wingless target genes (Korkut et al., 2009). Later studies revealed that WLS is present on exosomes isolated from medium conditioned by *Drosophila* S2 cells (Beckett et al., 2013; Gross et al., 2012; Koles et al., 2012) and that RAB11 contributes significantly to these exosomes (Beckett et al., 2013; Koles et al., 2012). Because presynaptic RAB11 knockdown prevents the transfer of Wingless across the NMJ, it was suggested that WLS and Wingless could be loaded together on exosomes that would then traverse the NMJ (Koles et al., 2012). Other studies have also suggested a role for exosomes in neuronal development and function (Lai and Breakefield, 2012), including axon pathfinding (Campbell and Peterson, 1993) and the regulation of myelination (Bakhti et al., 2011).

Whether exosomes contribute to the spread of Wingless along the imaginal disc epithelium remains a matter of debate. One study showed that overexpressed tagged WLS is released from secreting cells and colocalises with CD63-GFP punctae outside the expression domain, suggesting that this multipass protein can be released on exosomes (Gross et al., 2012). However, only a small proportion of extracellular Wingless colocalised with 'secreted' WLS, implying that these proteins are exported on different exosomes. This observation raises the question of whether, in this tissue, the release of WLS is relevant to the spread of Wingless, especially in light of the fact that such release has only been observed upon WLS overexpression. To identify proteins that contribute to Wingless secretion on exosomes, RNAi against genes encoding exosome-associated proteins was performed in S2 cells and the effect on Wingless secretion into the medium assessed. This identified the R-SNARE YKT6 (Gross et al., 2012). Crucially, YKT6 knockdown in imaginal discs led to a reduction in extracellular Wingless and the number of CD63-GFP exosomes, as well as a loss of expression of *senseless*, a Wingless target gene in this tissue (Gross et al., 2012). These results suggest that YKT6 could regulate the formation of Wingless-carrying exosomes. However, as YKT6 is known to function in endoplasmic reticulum-to-Golgi transport (Zhang and Hong, 2001), it will be important to demonstrate that the effect of YKT6 knockdown is not merely due to interference with general secretion.

Another potential means of reducing exosome formation, at least in cultured S2 cells, is to knock down RAB11 (Beckett et al., 2013; Koles et al., 2012). However, the expression of a transgene expressing RNAi against RAB11 in imaginal discs was shown to

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have no impact on the distribution and activity of Wingless (Beckett et al., 2013). This led the authors of this study to conclude that, contrary to the conclusion of Gross et al. (2012), exosomes are not important for the spread of Wingless in the wing imaginal disc. However, this conclusion too is subject to caveats. First, complete RAB11 suppression could not be achieved without causing cell lethality and it is conceivable that residual activity is sufficient to load Wingless on exosomes. Second, the lack of phenotype following RAB11 knockdown could be explained by the activity of a redundant Rab protein expressed in discs but not in S2 cells. An additional argument against a contribution of exosomes to the spread of Wingless comes from the observation that tagged WLS expressed at physiological levels does not transfer from cell to cell (Beckett et al., 2013). However, one cannot exclude the possibility that Wingless could be loaded on exosomes independently of WLS. It is clear from tissue culture experiments that exosome-associated Wingless does not account for all total secreted Wingless (Beckett et al., 2013; Gross et al., 2012). According to one estimate in cultured cells, it may only account for 20-40% of total signalling activity (Beckett et al., 2013), suggesting that a significant amount of active WNT can be released in an exosome-independent manner. Whether exosomes are required for full WNT activity *in vivo* remains difficult to ascertain because of the lack of tools to prevent exosome formation specifically (discussed below).

Extracellular vesicles and Hedgehog signalling

Exosomes have also been implicated in the secretion, diffusion and signal transduction of another class of another lipid-modified ligand, those of the Hedgehog (HH) family of secreted proteins. Only one Hedgehog protein is encoded by the *Drosophila* genome whereas vertebrates have three paralogues: Sonic hedgehog (SHH), Indian hedgehog (IHH), and Desert hedgehog (DHH) (Echelard et al., 1993). Whereas WNTs only carry palmitoleate on an internal serine residue, two lipids are appended to all HH proteins – cholesterol at the C terminus and palmitate at the N terminus (Chamoun et al., 2001; Lee et al., 1994; Porter et al., 1995). Preventing HH palmitoylation, e.g. by mutating the target cysteine, results in strong developmental defects, indicating that palmitate modification is required for HH signalling activity (Chamoun et al., 2001; Chen et al., 2004; Lee and Treisman, 2001; Micchelli et al., 2002). Evidence suggests that cholesterol is less important for signalling but could play an important role in determining the range over which HHs act. In the absence of the cholesterol moiety, high threshold target gene expression is reduced in intensity and range (Callejo et al., 2006; Dawber et al., 2005; Gallet et al., 2006) whereas low threshold targets continue to be expressed, although there is disagreement as to whether the range is extended or reduced (Callejo et al., 2006; Chamoun et al., 2001; Dawber et al., 2005; Gallet et al., 2006; Lewis et al., 2001; Li et al., 2006). Cholesterol could contribute to long-range action by stimulating the formation of micelles (Goetz et al., 2006; Zeng et al., 2001), by promoting integration into long-range transport carriers such as lipophorin particles (Panakova et al., 2005), or by stimulating interactions with glypicans, which would stabilise HH in the extracellular space (Avanesov et al., 2012; Callejo et al., 2006; Glise et al., 2005; Gorfinkiel et al., 2005; Sanchez-Hernandez et al., 2012).

Importantly, it has been shown that, in *Drosophila* embryos, the cholesterol moiety ensures packaging onto large punctate structures (LPSs), which can be observed over a relatively long range in the extracellular space (Gallet et al., 2003). Without the cholesterol modification, HH is no longer found on LPSs and can only diffuse over a short range. Although these LPSs were never further characterised, recent results suggest that they could represent exosomes or microvesicles. Similarly sized HH-containing particles colocalise with both MVB and exosomal markers, including CD63-GFP, in wing imaginal discs, and knocking down ESCRT components leads to insufficient HH secretion and target gene induction (Gradilla et al., 2014; Matusek et al., 2014). Moreover, both endogenous and overexpressed MVB components that are released into the extracellular space are captured by an endocytosis-deficient form of Patched (Ptc1130X) known to sequester extracellular HH at the cell surface (Matusek et al., 2014). In a separate study, Gradilla et al. (2014) showed that knockdown of α-SMase, which has been implicated in the formation of ESCRT-independent exosomes (see above), affects HH secretion. Therefore, both ESCRT-dependent and ESCRT-independent exosomes could contribute to HH secretion in wing imaginal discs.

Extracellular vesicles purified from insect cell lines overexpressing HH account for approximately 50% of HH signalling activity (Gradilla et al., 2014; Matusek et al., 2014). Moreover, perturbation of ESCRT proteins *in vivo* causes a decrease rather than complete loss of HH signalling target gene expression. These observations have led to the suggestion that only a proportion of Hedgehog is released on ESCRT-dependent EVs. However, both observations on which this conclusion rests are subject to caution. First, the unpolarised cells used for the culture assays do not provide an accurate model for the intricate apical-basal trafficking events that take place in imaginal discs cells (Burke et al., 1999; Callejo et al., 2011; Gallet et al., 2003; Yamazaki et al., 2016). Second, only partial perturbation of ESCRT is possible *in vivo* because complete and sustained inhibition would be cell lethal. Therefore, it remains to be determined whether ESCRT-dependent EVs account for all HH release, whether alternative spreading mechanisms are at work or whether exosomes contribute at all. Indeed the possible involvement of exosomes is contradicted by the earlier findings that HH does not colocalise with CD63-GFP and instead is found on lipoprotein particles (Panakova et al., 2005). One is left with the untidy proposition that EVs, lipoprotein particles and/or cytonemes could all contribute to the spread of HH. Irrespective of the level of contribution of EVs to the spread of HH, the nature of HH-containing EVs remains a subject of disagreement; Matusek et al. (2014) have suggested that they are microvesicles/ectosomes, not exosomes, as HH accumulates at the apical membrane upon ESCRT depletion, not at an endosomal site. However, this suggestion is at odds with results suggesting that HH must be endocytosed and targeted to MVBs before being secreted for long-range transport (Callejo et al., 2011; D'Angelo et al., 2015; Parchure et al., 2015). Moreover, in cultured cells, a form of HH that cannot be endocytosed is not secreted (Parchure et al., 2015). These data suggest that HH needs to be internalised before being released on EVs, which are therefore unlikely to be microvesicles/ectosomes.

Much of the evidence for the involvement of EVs in HH release comes from work with *Drosophila*. Studies of vertebrate limb buds have suggested that the main vertebrate Hedgehog, Sonic Hedghog (SHH) assembles in micelles for long-range transport in the extracellular space (Zeng et al., 2001) whereas, in the node of developing chick embryos, SHH has also been associated with exosome-like structures termed 'nodal vesicular parcels' (NVPs) (Tanaka et al., 2005). Whether NVPs are true exosomes remains to be demonstrated. SHH has also been shown to be secreted on exosomes from cultured human and primary chick notochord cell lines. In these contexts, two populations of exosomes – β-integrin positive and Lrp2/proteoglycan-4 enriched – with differing signalling efficiencies were detected (Vyas et al., 2014), perhaps indicating that exosomes of

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Table 1. Currently available means of interfering with exosome formation

Complex targeted	RNAi or DN used*	References	Effect	Caveats/other effects
Small GTPase	RAB11	Koles et al., 2012	Reduced release of WLS-containing vesicles from S2 cells; reduced postsynaptic WLS at neuromuscular junction	RAB11 regulates endocytic recycling; regulates membrane delivery during cytokinesis; participates in epithelial cell polarisation; regulates transcytosis of certain cargo; may be redundant with other Rabs
		Beckett et al., 2013	Reduced exosome release by S2 cells; no effect on Wingless gradient in imaginal discs	
		Gross et al., 2012	Lethal	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	RAB35	Beckett et al., 2013	No effect on exosome release from S2 cells	RAB35 regulates endocytic recycling; regulates endosomal trafficking of synaptic vesicles; may be redundant with other Rabs
		Koles et al., 2012	Reduced release of WLS-containing vesicles from S2 cells	
		Gross et al., 2012	No effect on wing patterning	
		Parchure et al., 2015	Reduced HH secretion in S2 cells	
	RAB27	Koles et al., 2012	Reduced release of WLS-containing vesicles from S2 cells	RAB27 is specific to exosome secretion; may be redundant with other Rabs
		Parchure et al., 2015	Reduced HH secretion in S2 cells	
	RAB10, RAB14, RAB6, RAB8	Gross et al., 2012	No effect on wing patterning	RAB10, 14, 6 and 8 are involved in a range of intracellular trafficking pathways
	ARF79F	Koles et al., 2012	Reduced release of WLS-containing vesicles from S2 cells	ARF79F is involved in intra-Golgi transport
	SAR1	Gross et al., 2012	Notching of wing margin	SAR1 is involved in ER-to-Golgi transport
ESCRT	ALIX	Matusek et al., 2014	Phenotype caused by HH overexpression partially suppressed	ESCRT is involved in the MVB degradative pathway and micro-vesicle formation; involved in cytokinesis; may be redundant in exosome formation
		Matusek et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Parchure et al., 2015	Reduced HH secretion and signalling in S2 cells	
	TSG101	Matusek et al., 2014	Phenotype caused by HH overexpression partially suppressed	
		Matusek et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	VPS22 (also known as SNF8)	Matusek et al., 2014	Lethal	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	CHMP1	Matusek et al., 2014	Phenotype caused by HH overexpression partially suppressed	
		Matusek et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	VPS24 (also known as CHMP3)	Matusek et al., 2014	Phenotype caused by HH overexpression partially suppressed	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	SHRUB (also known as CHMP4)	Matusek et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Parchure et al., 2015	Reduced HH secretion and target gene expression in imaginal disc	
	HRS	Beckett et al., 2013	No effect on exosome release from S2 cells	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Parchure et al., 2015	Reduced HH secretion and target gene expression in imaginal disc	

Continued

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Table 1. Continued

Complex targeted	RNAi or DN used*	References	Effect	Caveats/other effects
	VPS28	Beckett et al., 2013	No decrease in exosome levels in medium from S2 cells	
		Matusek et al., 2014	No reduction in target gene expression in imaginal disc	
	VPS4	Gross et al., 2012	Notching of wing margin	
		Matusek et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
		Parchure et al., 2015	Reduced HH secretion and target gene expression in imaginal disc	
V-ATPase	V-ATPase subunits 68.2, 26, 55, SFD	Koles et al., 2012	Reduced release of WLS-containing vesicles from S2 cells	V-ATPase is involved in endosomal maturation, acidification and trafficking
	V-ATPase 55kD B subunit	Gross et al., 2012	Mild notching of wing margin	
	V-ATPase subunits 100-2, C39	Gross et al., 2012	No effect on wing patterning	
SNAREs	Syntaxin 1A	Koles et al., 2012	Reduced release of WLS-containing vesicles from S2 cells; decrease in postsynaptic WLS at *Drosophila* neuromuscular junction	SNAREs are involved in fusion at many sites in the secretory and endocytic pathways
		Beckett et al., 2013	No decrease in exosome levels in medium from S2 cells	
	YKT6	Gross et al., 2012	Notched wing and reduced WG gradient and target gene induction	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	Synaptobrevin	Koles et al., 2012	Reduced levels of WLS vesicle release in S2 cells	
Other	Flotillin 1/Flotillin 2	Koles et al., 2012	Reduced levels of WLS vesicle release in S2 cells	Flotillin 1 and 2 are implicated in endocytosis, signal transduction and the cortical cytoskeleton
		Beckett et al., 2013	No decrease in exosome levels in medium from S2 cells	
		Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	
	Tetraspanin 42El, 86D, 96F	Gross et al., 2012	No effect on wing patterning	Tatrasplanins 42El, 86D, 96F are implicated in endocytic trafficking and growth factor signalling
	αSMase	Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	αSMase regulates lipid metabolism and hence various signalling pathways
	TAT-5	Gradilla et al., 2014	Reduced HH secretion and/or target gene expression imaginal disc	TAT-5 is involved in endocytosis and Golgi-to-ER transport
	Myosin 5	Koles et al., 2012	Reduced levels of WLS vesicle release in S2 cells; reduced postsynaptic WLS at neuromuscular junction	Myosin motor is needed for transport of vesicles and organelles

*Proteins that have been inhibited/knocked down to reduce exosome formation. The effect of such treatments on signalling by Hedgehog and Wingless in *Drosophila* imaginal discs or at the neuromuscular junction are listed, along with possible caveats.
DN, dominant-negative protein.

distinct composition and activity might exist. Unfortunately, most studies carried out to date have not been able to distinguish these different exosome classes in functional assays mainly because of over reliance on suppression of the MVB machinery and the ensuing pleiotropy to inhibit exosome formation.

An alternative genetic model system for investigating the roles of exosomes in development is the worm *Caenorhabditis elegans*. Although *C. elegans* does not have true HH orthologues, its genome encodes a number of HH-related proteins. Interestingly, these proteins are released on apical exosomes in an MVB-dependent manner (Liegeois et al., 2006). This process requires the V0-ATPase but not in a manner that requires proton-pump activity. The V0-ATPase seems to aid the docking of MVBs onto the plasma membrane, possibly through formation of V0 trans-complexes between MVB- and membrane-localised V0-ATPase. The role of V0-ATPase in the secretion of bona fide HH orthologues is yet to be tested. Another protein implicated in EV formation in *C. elegans*, though not in the context of signalling, is the P-4 ATPase, the loss of function of which causes large-scale shedding of EVs (Wehman et al., 2011). Even though these vesicles arise at the plasma membrane, their formation requires the ESCRT complexes and RAB11. One possibility is that loss of P-4 ATPase causes the accumulation of anionic phosphatidylinositols on the inner leaflet of the plasma membrane (Wehman et al., 2011). This could lead to the formation of anionic microdomains to which ESCRT-0 would bind (Henne et al., 2011) and then scaffold the formation of EVs. These observations could be relevant to HH function because the loading of HH onto EVs in the *Drosophila* wing disc has been proposed to originate at the plasma membrane rather than endosomes (Matusek et al., 2014). Indeed, the *Drosophila* TAT-5 homologue has been

shown to regulate HH secretion and signalling (Gradilla et al., 2014).

Exosomes as carriers of other developmental and tumorigenic signals

By virtue of their membranous nature, exosomes are natural carriers for membrane-associated signalling proteins such as WNTs and HHs. However, exosomes have also been implicated in signalling by transmembrane ligands of the Notch signalling pathway, in the context of vascular development and angiogenesis. The Notch ligand Delta-like 4 (DLL4) can be packaged into endothelial cell-derived exosomes and transferred to other endothelial cells and incorporated into their cell membrane, resulting in inhibition of Notch signalling and a loss of Notch reporter activity (Sheldon et al., 2010). Therefore, in this example, exosomes are possible carriers of a transmembrane ligand.

So far, exosomes have been implicated in only three classes of developmental signals (WNT, HH and Notch). But in the context of cancer biology, exosomes seem to modulate a wider range of signalling pathways. The ability of tumours to prime other tissues for metastasis is an emerging theme in cancer biology, and exosomes appear to be important mediators of this phenomenon (see Box 1) by transferring signalling molecules or modulators of signalling (Rak, 2015). For example, exosomes derived from mesenchymal stem cells (MSCs) promote angiogenesis by activating the extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 MAPK pathways (Zhu et al., 2012). Exosomes derived from colon cancer cells enhance growth through the transfer of oncogenic KRAS to recipient cells (Demory Beckler et al., 2013). Similarly, melanoma-derived exosomes transfer the receptor tyrosine kinase MET to bone marrow progenitors, programming them to support tumour vasculogenesis, invasion and metastasis (Peinado et al., 2012). Recently, pancreatic ductal adenocarcinomas have been found to produce exosomes that induce liver cells to secrete transforming growth factor β (TGFβ), which in turn contributes to pre-metastatic niche formation (Costa-Silva et al., 2015). How such exosomes could be targeted or exploited in anti-cancer therapies requires further investigation.

Conclusions

Although the body of literature suggesting a requirement for exosomes in developmental signalling grows, questions remain regarding whether the observed phenotypes are due to perturbation of exosome function or to pleotropic/collateral effects. As a result, there is currently no consensus on how long-range diffusion of HH and WNTs is mediated *in vivo*. Exosomes (Gradilla et al., 2014; Gross et al., 2012; Parchure et al., 2015), microvesicles (Matusek et al., 2014), lipophorin particles (Panakova et al., 2005) and cytonemes (Bischoff et al., 2013; Huang and Kornberg, 2015; Stanganello et al., 2015) have all been suggested to contribute, perhaps to different extents in different tissues. The absence of a means to interfere with these processes, exosome formation in particular, without affecting general cellular functions constitutes a major impediment to further progress. The tools used so far to perturb cytonemes have a deleterious effect on the actin cytoskeleton, and studies of the role of exosomes have relied on interfering with MVB formation or Rab and SNARE function. These protocols are not only likely to affect cellular health (e.g. by preventing lysosomal degradation) but also will probably affect signalling independently of exosome formation (e.g. by causing mis-trafficking of receptors). It is clear, therefore, that the most pressing challenge is to devise new tools to manipulate exosomes without perturbing other cellular processes. Furthermore, as there could be many types of exosomes, we must be ready for the possibility that no single protein or pathway alone controls the formation of all exosomes. Indeed, so far, it appears that a suite of proteins might be involved (summarised in Table 1). An alternative to knocking down essential components of exosome biogenesis could thus be to manipulate the sorting motifs that target specific signalling molecules to ILVs/exosomes. This could prevent their loading onto exosomes while avoiding pleotropic effects. However, such motifs remain to be identified. Moreover, there is a possibility that the lipids moieties on HH and WNTs could act as exosome targeting signals, which would make it difficult to separate loading on exosomes from signalling activity. In conclusion, although exosomes could provide a neat solution to the solubility problem of lipid-modified signalling proteins, a direct demonstration of their involvement is still required and the characterisation of the cellular mechanisms that specifically govern exosome biogenesis needs further investigation.

Competing interests

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