Heparanase involvement in exosome formation

Guido David[1,2] and Pascale Zimmermann[1,2]

1 Centre de Recherche en Cancérologie de Marseille (CRCM), Equipe labellisée LIGUE 2018, Aix-Marseille Université, Marseille F-13284, France and Inserm U1068, Institut Paoli-Calmettes, and CNRS UMR7258, Marseille F-13009, France

2 Department of Human Genetics, KU Leuven, Leuven, B-3000 Belgium

ABSTRACT

Exosomes are secreted vesicles involved in signaling processes. The biogenesis of a class of these extracellular vesicles depends on syntenin, and on the interaction of this cytosolic protein with syndecans. Heparanase, largely an endosomal enzyme, acts as a regulator of the syndecan-syntenin-exosome biogenesis pathway. The upregulation of syntenin and heparanase in cancers may support the suspected roles of exosomes in tumor biology.

KEYWORDS: ALIX, ESCRT exosome, exosomal cargo, heparan sulfate, heparanase, PDZ domains, syndecan, syntenin, tumor progression

Abbreviations

ALIX ALG-2-interacting protein X

CD cluster of differentiation

CTF C-terminal fragment

ESCRT endosomal-sorting complex required for transport

EV extracellular vesicle

ILV intraluminal vesicle

MVB multivesicular body

PDZ postsynaptic density 95/disc-large/zona occludens

SDC syndecan

Important messages, inserted into an envelope

Exosomes are small vesicles of endosomal origin, composing part of the complex collection of extracellular vesicles (EVs) that cells secrete. They contain various membrane and cytoplasmic components (i.e. membrane lipids and receptors, small GTPases, mRNAs and ncRNAs, etc) commonly designated as cargo, with a composition that reflects the state of the cell of origin (Skog *et al.*, 2008). Pending on the nature of that cargo, the presentation of these vesicles to 'recipient' cells can sometimes 'reprogram' the latter (Valadi *et al.*, 2007). Exosomes are therefore thought to play an important role in intercellular communication (Simons and Raposo, 2009; Colombo *et al*., 2014). This notion stems mostly from the field of cancer cell biology. For example, tumor-derived exosomes stimulate the formation of a pre-metastatic niche (Peinado *et al.*, 2012), and exosomal communication between cancer-associated fibroblasts and the primary tumor stimulates breast cancer cell motility and metastasis (Luga *et al*., 2012). Yet, the potential impact of exosomes extends far beyond cancer, including maintaining the stemness of progenitor cells and their participation in processes of tissue regeneration, inflammation and neurodegeneration (Théry *et al*., 2002; Fevrier *et al.*, 2004; Rajendran et al., 2006; Hergenreider *et al.*, 2012; Schneider and Simons, 2013; Schiro *et al.*, 2014). Several recent contributions provide an in depth review on the biology of EVs (Tkach and Théry, 2016; van Niel *et al*., 2018).

The making of an exosome

The mechanisms that control the biogenesis of exosomes and the sorting of specific cargo into these vesicles are only partially understood. Exosome biogenesis begins with the invagination of the plasma membrane leading to the formation of primary endocytic vesicles, and the fusion of these vesicles with each other to create the early endosomal compartment. While early endosomes mature into late endosomes, exchanging RAB5 for RAB7 and retaining a select subset of the endocytosed cargo they contain, a second invagination of the endosomal membrane occurs (but this time away from the cytosol) leading to the formation of so-called intraluminal vesicles (ILVs). Late endosomes that contain multiple (up to 30) ILVs are designated as multivesicular endosomes/bodies (MVE/MVBs). When MVBs fuse with

lysosomes, their cargo, including their ILVs, is degraded. However, late endosomes and MVBs can also fuse with the plasma membrane, releasing their ILVs into the extracellular space as exosomes (Raposo and Stoorvogel, 2013; Kowal *et al.*, 2014).

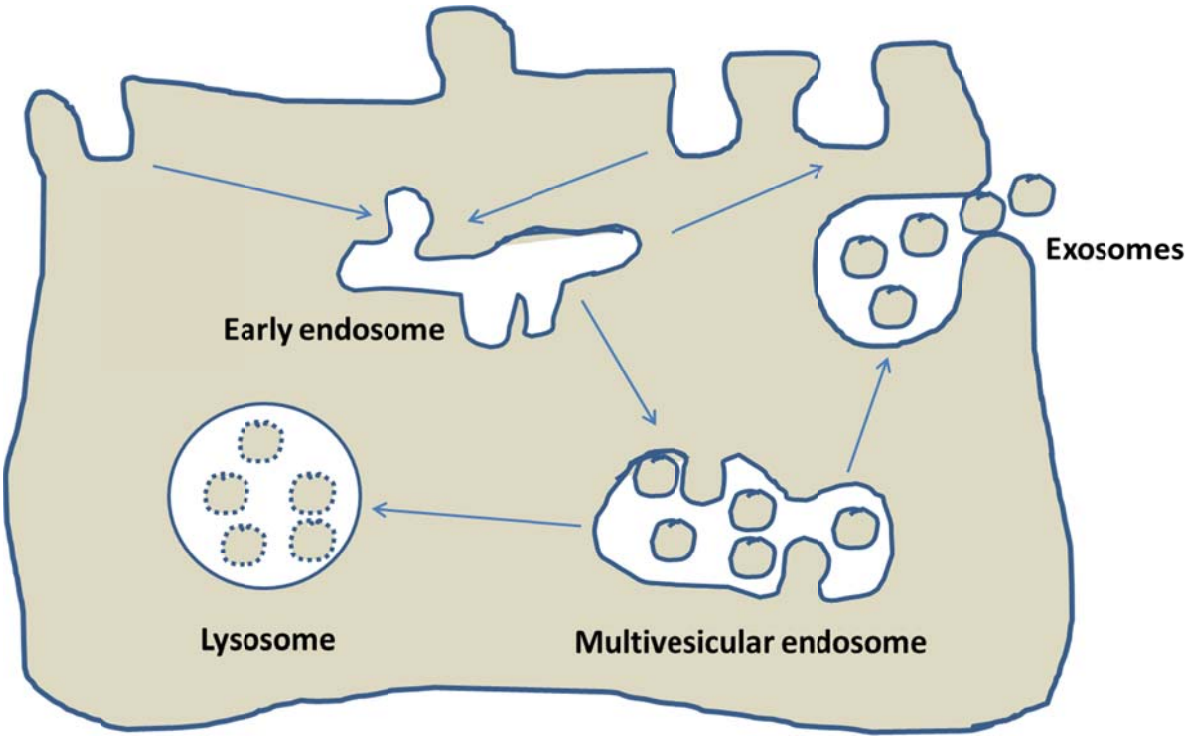

Figure 1. Exosome biogenesis. Intraluminal budding of the limiting membrane of endosomes creates intraluminal vesicles (ILVs). Endosomes that contain ILVs can fuse with lysosomes, but can also fuse with the plasma membrane, releasing their ILVs into the extracellular space as exosomes. Note that, as they result from two consecutive membrane bending events of opposite polarity (the first towards, the second away from the cytosol), ILVs and exosomes have the same surface topology as whole cells (cytosol/inside 'in', cell surface/outside 'out').

Several cellular components that participate in the control of intraluminal budding have been identified. Upon recruitment from the cytosol, the endosomal-sorting complex required for transport (ESCRT) machinery sorts internalized membrane proteins into specific membrane domains, induces the 'inward' budding of these domains (away from the cytosol, into the lumen of the endosome), and mediates membrane abscission to form ILVs (Hurley and Hanson, 2010; Wollert and Hurley, 2010; Henne *et al.*, 2011). The ESCRT machinery is composed of 4 multi-protein sub-complexes, of which ESCRT-0, -I and -II recognize and

sequester membrane cargo at the endosomal delimiting membrane, while ESCRT-III drives membrane budding and actual scission of intraluminal vesicles. It is important to note that originally the ESCRT machinery was found to drive the sorting of ubiquitin-conjugated membrane proteins into vesicles that bud into the lumen of a distinct set of MVEs that ultimately fuse with lysosomes rather than with the plasma membrane, resulting in the degradation of their vesicular contents (Raiborg and Stenmark, 2009). Sorting of proteins into exosomes, however, appears to occur independently of cargo ubiquitination (Buschow *et al*., 2009; Babst, 2011), and only a selected number of ESCRT components appear involved in exosome formation (Baietti *et al*., 2012; Colombo *et al.*, 2013). It is also important realizing that the mechanisms of cargo-sorting and membrane-budding and abscission are intimately intertwined. Clearly, also lipids have important and pleiotropic effects. That includes phosphoinositides, and in particular a role of these lipid signalling-intermediates (and thus of signalling processes) in recruiting ESCRT components with sorting functions to endosomal membranes (Raiborg *et al.*, 2001). Other studies have pointed to ceramide (Trajkovic *et al*., 2008) and other lipids (i.e. lysobisphosphatidic acid and phosphatidic acid) as mediators of ILV and exosome biogenesis (Laulagnier *et al*., 2004; Matsuo *et al*., 2004; Ghossoub *et al*., 2014; Egea-Jimenez and Zimmermann, 2017). Possibly these lipids are driving the lateral segregation of cargo into specialized endosomal membrane regions able to bend inwards. A more extensive and in depth review of what is known about the mechanisms involved in the biogenesis of these particular membrane domains and organelles can be found in several recent reviews (Hanson and Cashikar, 2012; Yang and Gould, 2013; Hurley, 2015; Schöneberg *et al*., 2017; Mathieu *et al*., 2019).

The reception of an exosome

Once released into the extracellular space, exosomes can reach recipient cells and present or deliver their contents to elicit functional responses. Exosome-mediated intercellular communication requires docking of the vesicles at the plasma membrane, followed by the activation of surface receptors and signalling, or their fusion with target cells, at the cell surface or in endosomal compartments, following internalization (French *et al.*, 2017). For example, exosomes may remain bound to the cell surface via integrins and from there activate intracellular signalling pathways, initiating cell migration (Sung *et al.*, 2015; Purushothaman *et al.*, 2016). Exosomes may also be internalized, by multiple pathways: i.e. receptor mediated

endocytosis, phagocytosis and macropinocytosis (Feng *et al.*, 2010; Mulcahy *et al.*, 2014; Nakase *et al.*, 2015). In general, the ways exosomes interact with cell surfaces and vesicular cargo is transferred to other cells remain poorly understood. These processes are no doubt complex and likely depend on the cargo of the exosomes, and thus the compositions of the exosome-donor cells, and on the identity of the recipient cells, with their particular repertoire of receptors and endo/phagocytic properties. The fate of endocytosed exosomes might be quite different in professional macrophages and in epithelial cells, but the general principles of exosome trafficking are likely shared. It is thought that along some of these paths, under some conditions, internalized exosomes fuse with the limiting membrane of the endosome, delivering their cytosolic cargo (i.e. RNAs) to the cytosol of the recipient cell, in a process that topologically represents the exact reverse of their biogenesis. It would seem that such 'back-fusion' should be of a magnitude sufficient for reaching the stoichiometries that are needed for the delivered cargo having meaningful effects (e.g., for mIRs to effectively target RNAs). Possibly transfers of the required magnitude occur through specific vesicles or only under certain specific conditions.

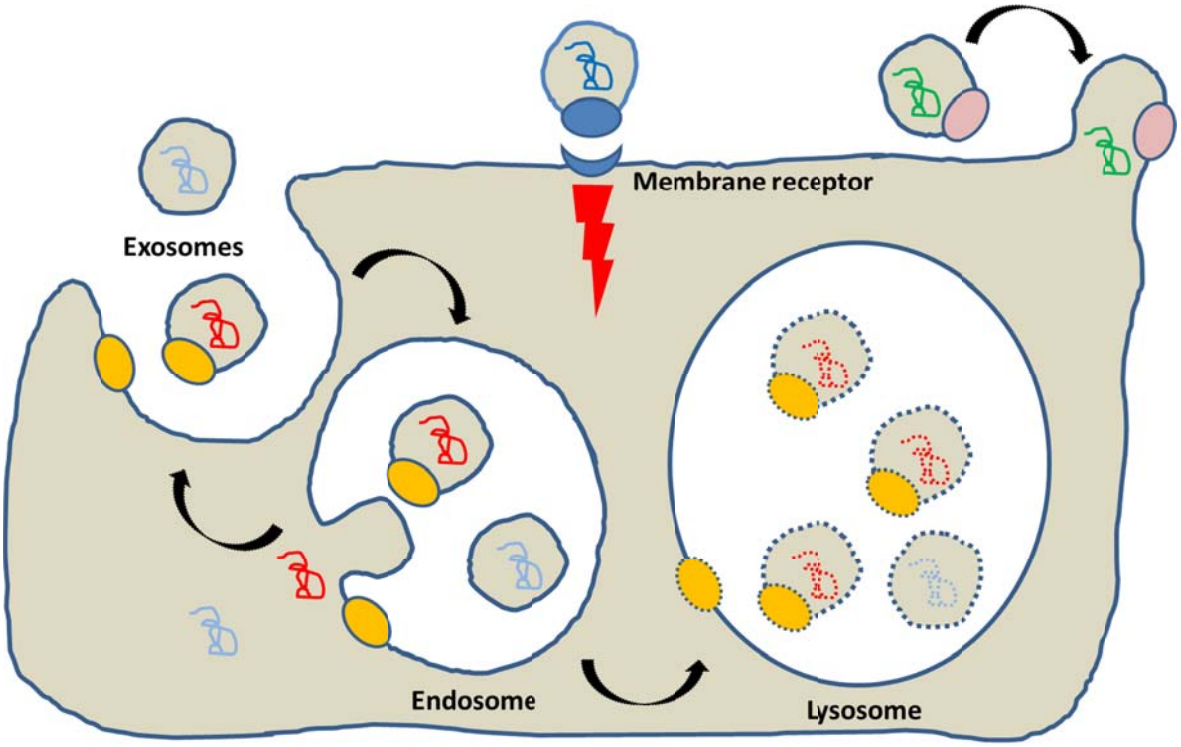

Figure 2. Exosome reception. In the recipient cell (which can be the producing cell itself), exosomes will bind to the cell surface and from there can undergo various fates. Exosomes can activate membrane receptors and initiate signaling directly from the cell surface. Internalization will introduce exosomes into the endosomal pathway. Ultimately, these will

reach multivesicular endosomes, where they likely mix with endogenous intraluminal vesicles. Fusion of the multivesicular endosomes with the lysosome will lead to the degradation of these exosomes. Possibly, if not likely this is the major fate of any internalized exosome. Yet, exosomes docked either at the plasma membrane or at the limiting membrane of early and late endosomes can probably also 'back-fuse' with that membrane, releasing their intraluminal contents into the cytoplasm of the recipient. That process is currently poorly understood, but is of major importance for delivery of intraluminal cargoes such as microRNA (miRNA).

Virus-like vesicles, exosome-like viruses?

It should be clear that in many aspects both the biogenesis and secretion of exosomes and the ways by which these vesicles 'transduce' recipient cells is reminiscent of the life cycle of viruses. Moreover, some viruses exploit the host mechanisms of membrane bending and abscission to egress from cells. Human immunodeficiency virus-1 (HIV-1) and Equine Infectious Anemia Virus (EIAV), for example, exploit components of the ESCRT machinery to bud, directly from the plasma membrane or in MVBs (Strack *et al.*, 2003; Fujii *et al.*, 2007; Morita *et al.*, 2011; Hurley and Cada, 2018). The P(T/S)AP motifs and the LYPxnL motifs present in the late domain proteins of these retroviruses interact directly with, respectively, TSG101 (an ESCRT-I component) and with ALIX (an auxiliary component of the ESCRT machinery, bridging TSG101 in ESCRT-I and CHMP4 in ESCRT-III), and these interactions are essential for virus release. There is thus even a strong mechanistic analogy between viral budding and ILV/exosome formation. In addition, some viruses use the same mechanisms as extracellular vesicles, and sometimes these vesicles themselves to enter cells. It thus comes as no surprise that exosomes are often considered as 'natural, endogenous' viruses, and that the distinction between viruses and extracellular vesicles has even become somewhat 'semantic'. The concept was well formulated and summarized in the 'Trojan exosome' hypothesis, now already fifteen years ago (Gould *et al.*, 2003). More than ever this conceptual framework remains valid (Wurdinger *et al.*, 2012; Nolte-'t Hoen *et al.*, 2016). As we will discuss, also the link of heparanase to exosome biology will underscore this notion.

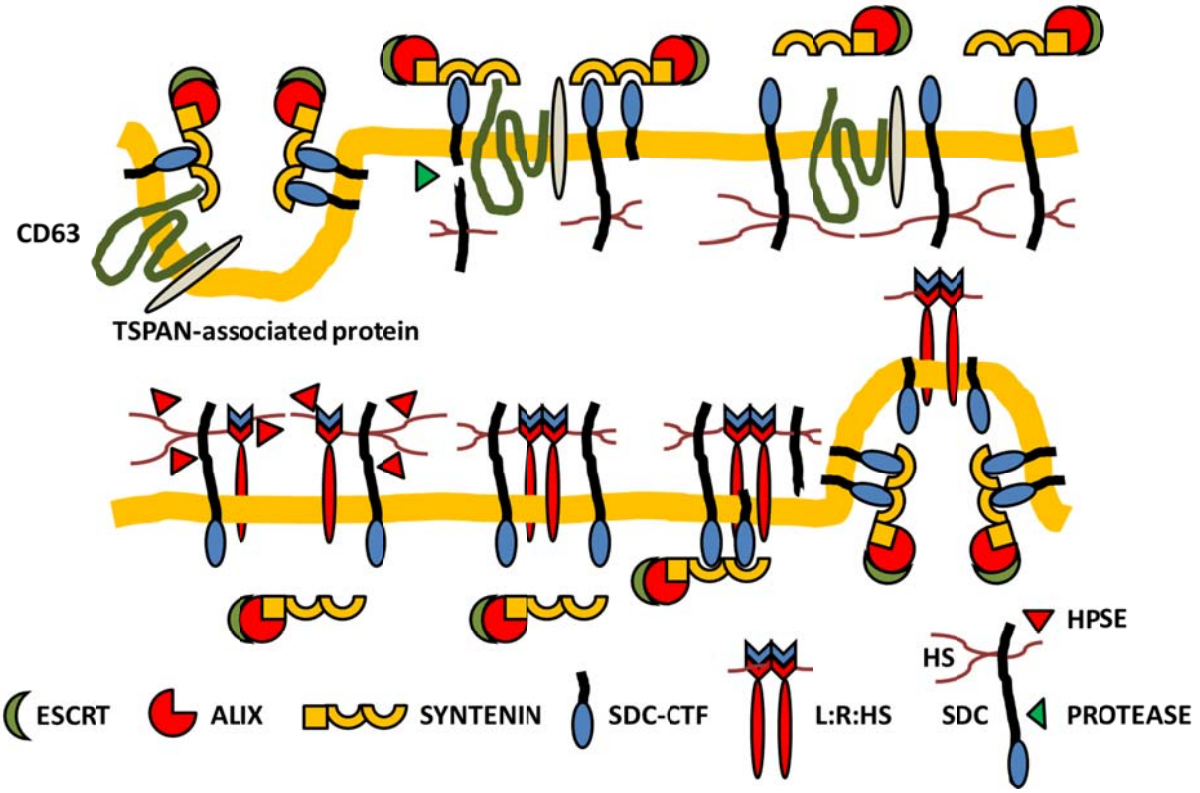

Figure 3. Heparanase 'activates' syndecan for exosome production. Syndecan and cargo bound to the heparan sulfate (HS) of syndecan (e.g. FGF-FGFR complexes assembled on the HS chains of syndecans; Ligand:Receptor:HS) are internalized by endocytosis. In endosomes, syntenin directly interacts with syndecans and the tetraspanin (TSPAN) CD63 via its tandem PDZ domains. During endosome maturation into late endosomes, syndecans (SDC) are trimmed by heparanase (HPSE) and undergo proteolytic cleavage of their extracellular part to generate a membrane-associated syndecan C-terminal fragment (SDC-CTF). These cleavages allow syndecans / syndecan C-terminal fragments to cluster, recruiting syntenin, and stimulate endosomal budding. The syntenin-mediated endosomal budding of syndecan and CD63, and of cargo associated with these proteins, also depends on the direct interaction of the N-terminal domain of syntenin with ALIX (an ESCRT accessory component), and on several ESCRT proteins. Heparanase does not stimulate all types of exosomes; it stimulates the exosomal release of syndecan C-terminal fragment, syntenin, ALIX and CD63, but has no effect on the release of exosomal flotillin, CD9, or CD81 (two other tetraspanins commonly found in exosomes).

Syntenin, adapting ESCRT machinery to endocytosed syndecans supports the biogenesis of exosomes

Former contributions from our laboratories strongly implicate the syndecan heparan sulfate proteoglycans and their cytoplasmic adaptor syntenin in the biogenesis of exosomes. The syndecans (SDCs) compose a family of type-1 membrane-spanning proteins, exposing heparan sulfate (HS) chains with versatile properties at the cell surface and an evolutionary highly conserved small intracellular domain (ICD) in the cytosol. HS has numerous ligands, including various morphogens, adhesion molecules and growth factors, e.g. Wnts, fibronectin and fibroblast growth factors (FGFs) to name a few (Fuster and Esko, 2005; Lindahl and Kjellen, 2013). HS plays an important role in the docking of these factors to cognate signalling receptors, e.g. the binding of FGF2 to FGF-Receptor1 (FGR1), qualifying the cell surface proteoglycans as 'co-receptors'. HS and SDCs in particular are also intimately involved in several processes of endocytosis and vesicular trafficking that depend on cellular context and type of ligand (Lambaerts *et al.*, 2009; Sarrazin *et al.*, 2011; Christianson and Belting, 2014). Yet, the biological effects of the SDCs do not solely rely on their HS chains. Direct interactions of their protein cores, in particular of their conserved transmembrane and cytoplasmic domains, but also of their ectodomains, are now well characterized (Beauvais and Rapraeger, 2010; Beauvais *et al.*, 2016). Of particular importance, in the present context, is that all SDCs feature a strictly conserved EFYA sequence at their cytosolic C-terminus. Syntenin is a protein that binds to the syndecan-ICD, via that C-terminal structure (Grootjans *et al.*, 1997). Syntenin is a small, 298 amino acid cytosolic protein that contains two PSD95/Dlg/zonula occludens 1 (ZO-1) (PDZ) domains in tandem, surrounded by a 100 amino acid N-terminal and a 25 amino acid C-terminal region. The two PDZ domains of syntenin are both necessary and sufficient for syntenin membrane localization and high-affinity interaction with SDCs (Grootjans *et al.*, 2000; Zimmermann *et al.*, 2001). Of note, the syntenin PDZ domains also interact with phosphatidylinositol 4,5-bisphosphate (PIP2) (Zimmermann *et al.*, 2002) and, pending on the activation of ARF6 and PIPK, syntenin-PIP2 interaction controls the endocytic recycling of SDCs and of SDC-associated complexes, i.e. FGF2-FGFR1 complexes, from late recycling endosomes back to the cell surface (Zimmermann *et al.*, 2005). Syntenin probably occurs in alternative 'open' and 'closed' configurations, likely involving intramolecular interactions between the N-terminal and C-terminal domains of the protein and potentially controlling its further intermolecular interactions and recruitment to cell surfaces. Importantly, the N-terminal domain of syntenin can directly interact with ALIX (Baietti *et al.*, 2012). This interaction occurs via three $LYPx_nL$ motifs present in the syntenin N-terminal domain that bind the ALIX 'V domain', and is thus reminiscent of the ALIX

interaction with late viral domains of HIV-1 and EIAV (see above). *In vitro* BIACore experiments indicate that recombinant SDCs, syntenin and ALIX proteins can assemble in a tripartite complex. In counterpart, *in cellulo* gain- and loss-of-function experiments indicate that SDCs, syntenin and ALIX work together in exosome formation and composition. The whole of the results suggests a model whereby the clustering of endocytosed SDCs is recruiting syntenin, and syntenin is adapting syndecans and syndecan-associated cargo to ALIX and ESCRT proteins (in particular the CHMP4 proteins of the ESCRT-III complex that bind ALIX), all working together in the budding and abscission of endosomal membranes to form ILVs and sequestering particular cargo in these ILVs (Baietti *et al*., 2012; Hurley and Odorizzi, 2012). For example, in the presence of FGF2, SDC, syntenin and ALIX control the exosomal release of FGFR1. They also control the exosomal release of CD63, a tetraspanin that is often used as a marker of exosomes, but not that of CD9 or that of flotillin-1 (both also often used as 'exosomal markers'). Of note, like syndecans, CD63 (but not CD9) also binds to syntenin, via a PDZ-BM (Latysheva *et al*., 2006). Importantly, the production of these SDC-CD63-syntenin exosomes depends on the HS chains of the syndecans and the participation of this HS in the lateral interactions of the syndecans with specific ligands (like FGFs) that lead to their clustering, and from there the recruitment of syntenin. At least in some types of cells and under some conditions, the SDC-syntenin-ALIX connection is an important path that controls up to 50% of the vesicles that are secreted by the cells. Possibly this relates to the versatility of the syndecans as co-receptors, and the multitude of signaling processes that require the assistance of HS. Importantly, the participation of SDC-syntenin-ALIX in ILV budding and exosome formation also depends on the activation of ARF6, but in this case on PLD2, an enzyme synthesizing phosphatidic acid, as ARF6 effector (Ghossoub *et al.*, 2014). Altogether, these observations poise syntenin as critical 'checkpoint' in the control of the trafficking of syndecan and syndecan-associated endosomal cargo, sending these back to the cell surface or to ILVs and exosomes. Of note, ILV formation, initiated by signaling, ultimately subtracts signaling receptors from the cytosol and terminates their contacts with cytosolic signal transducers and effectors. Thus, the SDC-syntenin connection potentially further extends the role of HSPGs in the control of signaling, far beyond their roles as co-receptors and involvement in signal initiation: sustaining signaling (by recycling) versus terminating signaling (via ILV formation) *in cis*, and transferring signaling cargo (via exosomes) for potential use *in trans*.

Heparan sulfate involvement in exosome internalization

Exosome internalization can be studied by confocal microscopy and flow cytometry, using labeled vesicles. For example, using vesicles marked by a fluorescent dye (PKH) with long aliphatic tails that are incorporated into the lipid membrane of the vesicles, in principle labeling all vesicles in the population, or using vesicles that are loaded with specific eGFP-cargo, potentially representing only a specific subset of the exosomes. This way, it can be shown that (in several different types of cells) exosome uptake is dose-dependent and saturable, vesicle accumulation increasing with incubation time and being inhibited by incubation at 4 °C and by the presence of excess, unlabeled exosomes. The group of Matthias Belting has demonstrated that such internalized exosomes co-localize inside cells with HS-epitopes and with cell-surface HSPGs of the syndecan and glypican type. Exosome uptake is significantly inhibited by added HS and heparins, in a dose-dependent way and in a specific manner, closely related chondroitin sulfate having no effect. In addition, multiple mutant cell types, deficient in enzymes involved in HS-synthesis and modification, provide genetic evidence of a receptor function of HSPG in exosome uptake. Similarly, enzymatic depletion of cell-surface HSPG (by treating recipient cells with bacterial heparinases, removing all HS from the cells) or pharmacological inhibition of endogenous PG biosynthesis (by xyloside) significantly attenuate exosome uptake. Although to a certain extent some intact HSPGs are sorted to and associate with exosomes, similar enzyme treatments of the vesicles suggest that exosome-associated HSPGs have no direct role in exosome internalization. Finally, isolated exosomes bind to heparin-substituted beads. Thus, added HS inhibits cellular uptake of exosomes through competition with cell-surface HSPGs for exosome binding. It is important to note that even in the presence of added heparin or HS-depletion from cells, significant uptake activity remained. Yet, on a functional level, exosome-mediated stimulation of cancer cell migration appeared to be significantly reduced in HS-deficient mutant cells, or by treating wild-type cells with heparin, lyase or xyloside. Thus cells use (the assistance of) HSPGs for internalizing exosomes and responding to these vesicles, which significantly extends the role of HSPGs as key receptors of macromolecular cargo (Christianson *et al*., 2013). Clearly, these data do not exclude the possibility that exosomes may also exert functional effects through alternative pathways, all or not involving exosome uptake. Given that several viruses have previously been shown to enter cells through HSPGs, these data further implicate HSPG as a convergence point during cellular uptake of endogenous vesicles and virus particles. Of note, specific HS modifications (generating structures based on 3-O-sulfate) have also been

implicated in productive viral infection, implying fusion and access of viral contents to the cytosol of the cells (Shukla *et al.*, 1999). Thus, by extension, possible additional roles for HS in exosome fusion are to be considered.

Interestingly, cells that are syntenin-deficient appear to 'resist' transduction by recombinant AAV *in vivo* and by recombinant retrovirus *in vitro* (Fares *et al*. 2017; Kashyap *et al.*, unpublished results). Compared to controls, syntenin-deficient cells internalize also lesser amounts of PKH-labeled exosomes. Strikingly, syntenin-deficient cells express lower amounts of HS at their cell surfaces, likely at least in part a reflection of the function of syntenin in SDC recycling. Over-expressing SDCs in syntenin-deficient cells markedly enhances the effectiveness of retroviral transduction. So does the re-introduction of wild-type syntenin, but syntenin mutants that are defective in either the recycling-function or budding-function of syntenin do not. The latter is particularly intriguing, and remains to be explained, but it might be noted that the PDZ-domains of syntenin bind also avidly to nectin-1 (Garrido-Urbani *et al.*, 2016), along with 3-O-sulfate-substituted HSPGs, one of the several receptors involved in the entry of HSV-1 into cells. Syntenin controls also the post-endocytic trafficking of oncogenic human Papillomaviruses (Gräßel *et al*., 2016). Although some of the current evidence remains largely conjectural, all these observations would seem to place syntenin both at the 'sending end' and at the 'receiving end' of exosome biology.

Heparanase activates the syndecan-syntenin-ALIX exosomal pathway

Heparanase is an endoglycosidase, cleaving heparan sulfate chains at internal sites, generating short HS fragments (of 10 to 20 residues). It is the only mammalian enzyme with such activity (Vlodavsky *et al*., 1999). The importance of heparan sulfate for both exosome production and clearance implies that heparanase might influence processes of exosomal exchange. As are exosomes, heparanase is strongly implicated in tumor invasiveness, angiogenesis and metastasis (Edovitsky *et al*., 2004). The notion receives also support from the emerging evidence for the implication of heparanase in viral infection, as exemplified by herpes simplex virus-1 (HSV-1), one of the first viruses shown to attach to cell surface heparan sulfate (HS) for entry into host cells. During a productive infection, the HS moieties on parent cells trap newly exiting viral progenies and inhibit their release. Yet, heparanase expression is upregulated upon HSV-1 infection, modifying the HS present at cell surfaces, facilitating viral

release. Thus, heparanase seems to act as a molecular switch for turning a virus-permissive 'attachment mode' of host cells to a virus-deterring 'detachment mode' (Hadigal *et al.*, 2015). Since many human viruses use HS as an attachment receptor, the heparanase-HS interplay may delineate a common mechanism for virus release (Takkar *et al.*, 2017). By extension, such scheme might also apply to exosomes.

Consistently, elevating heparanase expression in cells stimulates net exosome production and affects the composition of exosomes, enhancing the loading of these vesicles with cargo that potentially influences angiogenesis (Thompson *et al.*, 2013; Roucourt *et al.*, 2015). Experimentally, it is fairly easy to increase the levels of heparanase activity within cultured cells. When pro-heparanase is added to cells, the enzyme precursor is rapidly internalized and processed into active heparanase (Gingis-Velitski *et al.*, 2004; Vreys *et al.*, 2005). This conversion occurs in endosomes, where the enzyme normally remains localized (Zetser *et al.*, 2004). The opposite, fully suppressing endosomal heparanase activity in cells might be somewhat more complicated (at least with the inhibitors that are currently available), given that sera used for culturing cells contain substantial amounts of platelet heparanase, and given the long half-life of internalized heparanase. An increase in heparanase results in extensive trimming of the heparan sulfate on syndecan and also accelerates the endocytosis of syndecan. Exosomal levels of syntenin and CD63 increase markedly, but most striking is the increase in exosomal syndecan. Of note, most of that syndecan consists of C-terminal fragments (CTFs) that span the membrane but are devoid of any heparan sulfate (or chondroitin sulfate). Conversely, in cells that express high levels of heparanase, stable shRNA-mediated knockdown of the enzyme reduces the amounts of syntenin, CD63, and syndecan-CTFs present in exosomal fractions.

Importantly, the catalytic activity of heparanase is required and heparan sulfate must be provided by syndecan. Indeed, glypicans, heparan sulfate proteoglycans that are linked to the cell surface via glycosylphosphatidylinositol (and thus cannot directly interact with syntenin), cannot substitute for syndecan and restore the effect of heparanase on exosomes. Knockdown of the small GTPase RAB7 abolishes the heparanase-mediated increase in exosomal syntenin, syndecan CTFs, and CD63, confirming that heparanase affects the production of vesicles that are of endosomal origin, the operational definition of exosomes. Furthermore, heparanase stimulates the endosomal budding of syntenin and syndecan, and requires ALIX for these effects. Thus, heparanase is an activator of the syndecan-syntenin-ALIX pathway of exosome

biogenesis (Roucourt *et al.*, 2015). In contrast, using exosomes loaded with eGFP-syntenin and various recipient cells, added heparanase appears to have little or no effect on (syntenin) exosome uptake. Yet, this aspect has been less investigated, and, as already stated, might also depend on the donor and the repertoire of exosome-associated membrane proteins and cargo. Finally, and also of note, exosomal flotillin-1 and exosomal levels of CD9 and CD81, two tetraspanins also commonly used as exosomal markers, are not affected by heparanase. The specificity of the heparanase effect underpins the hypothesis of multiple different exosomal populations formed through specific biogenesis pathways, one of which is the syndecan-syntenin-ALIX pathway (Friand et al. 2015; Kowal *et al*., 2016).

Heparanase, integrating syndecan lateral associations and spatial constraints?

How might heparanase influence syndecan-dependent endosomal membrane budding? The initial clustering and introduction of the syndecans in endocytic pathways likely depends on the lateral associations of these molecules with ligand, explaining the need of HS that is present on syndecan. An important second concept is that syntenin is recruited to membranes by clustered 'bait', engaging both the PDZ domains of syntenin (Grootjans *et al.*, 2000; Zimmermann *et al*., 2001). Thus, reaching the local concentration of syndecan-ICD required for recruiting syntenin might require the remodelling of the syndecans by heparanase and ultimately their conversion into a membrane embedded C-terminal fragment that lacks HS. Finally, membrane budding and endosomal filling likely also imply a reduction of repulsive forces in the endosomal luminal space. Heparanase might have impact at several levels of such scheme (David and Zimmermann, 2015; Roucourt *et al.*, 2015; Stoorvogel, 2015). A first consideration is that lateral interactions engaging the HS chains of the proteoglycans sometimes depend on HS remodelling. In the case of HS-assisted FGF-FGFR signaling, for example, it is striking that bacterial heparitinase (Zhang *et al.*, 2001), and likewise mammalian heparanase (Kato *et al.*, 1998; Reiland *et al.*, 2006), can generate HS-fragments endowed with biological activity, where that activity is rare or even not present in the intact parent HS-chain. At least in the particular case of FGF2-FGFR1, crystal structures reveal that in order for two trimeric FGF-FGFR-HS complexes to assemble and confront one another in hexameric signaling units, the specific HS-structures that foster and stabilize the formation of FGF-FGFR complexes need to occupy a terminal position in each of the two HS chains that are engaged in the formation of a signaling unit (Schlessinger *et al.*, 2000). In other words,

HS-supported FGF-FGFR interfaces will bring together or cluster two different heparan sulfate chains, in opposing orientations, pending on occupying the 'end-structures' of these chains. In that scheme, pending on the presence of FGF and FGFR, heparanase, converting the HS chains on SDC into shorter chains with the required end-structures, leads to the clustering of SDCs. In short, heparanase potentially 'activates' the lateral HS-mediated associations of the syndecans, inducing the SDC clustering that can recruit syntenin. In that specific context, it might be worth reminding that FGF2 stimulates the production of exosomes that contain FGFR1 (along SDC-CTFs and syntenin), and that the down-regulation of syndecans or syntenin attenuates such effect of FGF2 (Baietti *et al.*, 2012). A variant on that theme may be provided by lacritin, an epithelial mitogen that activates PLD-mTOR (Wang *et al.*, 2006) and is linked to autophagy (Wang *et al*., 2013). This mitogen specifically binds to syndecan-1, and not syndecan-4, via heparanase-modified HS and the concomitant exposure of a binding site in the syndecan-1 core protein (Ma *et al.*, 2006; Zhang *et al*., 2013). Heparanase-modulated lateral syndecan associations might be initiated in endosomes, where heparanase resides, or, more likely, be initiated on syndecans that recycle from endosomes back to the cell surface (a process that is supported by PIP2-syntenin). Secondly, one might also have to consider the mirror aspects and side effects of these 'fatal attractions'. SDCs are present at cell surfaces in such high copy number, that they probably suffice to cover the entire cell surface (Yanagashita and Hascall, 1992). As the HS chains are higly negatively charged, HSPGs will probably tend to repel each other, and be 'locked' in their positions unless engaged and 'neutralized' by ligand. Having extended structures, native HS chains also potentially bind multiple ligands at the same time. Potentially such ligands have different mobilities, restricting allover HSPG and ligand mobility: for example, matrix-bound SDC, limiting the mobility and availability of growth factors and growth factor receptor complexes bound and assembled on that same SDC (Mali *et al.*, 1993). Heparanase, in contrast, leaves syndecan substituted with small heparan sulfate chains (possibly restricted to a single ligand) and thus more likely free to 'move around'. Consistent with the above notions of potential heparanase effects on SDC engagements and mobility, loading cells with heparanase, markedly shortening the length of the HS chains on syndecan-1, significantly accelerates the endocytosis of that proteoglycan (Roucourt *et al.*, 2015). Potentially, accelerated endocytosis is helping increasing the concentrations of endosomal syndecan up to levels required for recruiting syntenin. The possible importance of a reduction of the physical dimension of a syndecan becomes also particularly compelling when considering the dimensions of an ILV. The length of an extended native heparan sulfate chain (with a molecular weight of 40 kDa on

average) is close to 50 nm. With three chains of heparan sulfate per syndecan, likely projecting outwards and pointing away from each other, the diameter of a syndecan may be close to 100 nm, which is approximately the diameter of an ILV and exosome. Trimming of the heparan sulfate on syndecan may thus substantially increase the number of syndecan molecules that can be packed in defined membrane domains and thereby help create the local concentration of bait (syndecan cytosolic domains) that will allow recruiting syntenin and along with the syntenin also ALIX and ESCRTs. Conceivably, even in ligand-induced SDC complexes supported by trimmed HS (with still a mass of about 7-10 kDa), the SDC-ICDs may remain too far apart for recruiting syntenin. Thus, obtaining the degree of clustering or compaction of syndecan that is sufficient for recruiting syntenin might require the cleavage of the protein, leaving a SDC-CTF in association with the endosomal membrane. Possibly, the initially mixed oligomerizations of HS-substituted SDCs and HS-free SDC-CTFs (not subject to self-repulsion), in the end replaced by the oligomerizations of mainly SDC-CTFs, may stably recruit syntenin and ALIX. In that context, one might surmise that once also ESCRT-III is recruited, and that CHMP4 assemblies are surrounding the necks of the buds, HS becomes entirely dispensable for sequestring SDC-CTFs and syndecan-associated cargo in budding membranes. Conceivably, heparanase-mediated trimming of the HS on syndecan might facilitate the access of processing protease generating the SDC-CTF, potentially including metalloproteases and acid proteases. It is noteworthy, in that respect, that upregulation of heparanase also induces the shedding of syndecan-1, by metalloproteinase processing (Yang *et al.*, 2007), and, inversely, that heparanase-deficient animals show an upregulation of metalloproteinases (Zcharia *et al.*, 2009). It is not clear to what extent cell surface and endosomal activities are involved, but possibly up-regulation of processing protease can compensate for reduced protease-access. Furthermore, the speculative considerations made on molecular HSPG crowding at cell surfaces can also be made for endosomes, where, conceivably, internalized HSPGs might continue repelling each other, in the lateral plane of the limiting membranes, at the level of budding membranes, and between budding and limiting membranes. The most compelling spatial constraints would seem to occur at the level of the neck of the bud, an area of extreme membrane curvature, where ESCRT-III accumulates to mediate membrane fission (Schöneberg *et al.*, 2017). HS-persistence on limiting and budding membranes could thus potentially have a vesicle ‘back fusion’ effect. Inversely, HS-removal, possibly initiated at selected parts of the endosomal membrane, creating asymmetric distributions of mass across the bilayer and changes in molecular crowding, might have a ‘permissive’ effect. When endosomes are filling up with

multiple ILVs to form MVE, there might also be a general ‘need’ for further reducing the net or effective negative charge of the membranes of these compacting compartments. Such might again be achieved by neutralizing by ligand, likely enhanced by the acidification of the compartment, increasing the net positive charge of all proteins without affecting the negative charge of the HS, or, more effectively, by reducing the mass of the HS or removing the HS on these membranes altogether. In that respect, heparanase, fragmenting the HS, might also markedly accelerate the process of HS removal by exoglycosidases. Being directed to late endosomes and lysosomes by secretion and recapture, possibly heparanase can allow doing so at a stage during endocytosis, i.e. a time window or compartment, where ILVs can still be diverted from lysosomal degradation and secreted as exosomes (and sole exoglycosidases might come ‘too late’ to complete the membrane remodelling that is required).

Heparanase effects on exosomal cargo

It is interesting to note that, upon heparanase addition, the amounts of exosomal cargo that composes the direct 'bait' for the PDZ domains of syntenin (i.e. syndecan CTFs and CD63) continue to increase with increasing heparanase concentrations, but that the effect of heparanase on exosomal syntenin plateaus. These findings are in line with prior observations that the ratio of 'bait' or cargo to syntenin in exosomes is not constant (Baietti *et al.*, 2012). Indeed, the over-expressions of SDCs or CD63 markedly increase the levels of the corresponding SDC-CTFs or CD63 in exosomes, but do not affect the levels of syntenin and ALIX in these vesicles. Thus, while intraluminal budding/exosome formation appears to be triggered by the organization of syntenin bait (i.e. syndecan-CTFs and CD63) in structures of higher order, syntenin may adapt only to a part of the bait present in these organizations. This situation would again be similar to the incorporation of viral proteins in budding membranes, where, due to lateral associations between coat proteins, late domains are also functioning *in trans* and not all individual copies of the GAG-proteins that end up in the viral coats need to be provided with direct links to the budding machineries (Wills *et al.*, 1994). Similarly, a sizeable fraction, but not all of the syntenin-dependent cargo that ends up in exosomes might need to be directly linked to syntenin. Thus, where the cellular levels of syntenin may become limiting at some point (and exosome numbers might stagnate), heparanase might still increase the 'lateral association' or clustering of syndecans, further stimulating the incorporation of syndecans and syndecan-associated proteins in exosomes. In myeloma cells, for example,

heparanase stimulates the accumulation of syndecan-1 and of specific cargo such as hepatocyte growth factor and VEGF in exosomes (Thompson *et al*., 2013). Whereas syndecan-1 is a well-known marker of plasma cells and predominates in myeloma cells, more remarkably, also in MCF-7 and other cells the effects of heparanase are very marked for the exosomal levels of SDC1-CTFs. In comparison, effects on the levels of SDC4-CTFs are more moderate (Roucourt *et al*., 2015). Such differential effect of heparanase on exosomal syndecan might be context-dependent, but suggests differences between the syndecan family members that might relate to their differential subcellular localization, trafficking, heparan sulfate composition, access to heparanase or syndecan-cargo associations. In particular in terms of that last aspect, it might be important to remind about the increasing evidence for extracellular lateral syndecan associations that involve their core proteins: e.g. syndecan-4 interacting directly with EGFR (Beauvais *et al.*, 2010; 2016); the protein tyrosine phosphatase CD148 binding to a region proximal to the transmembrane domain of syndecan-2 (Whiteford *et al.*, 2011); the integrin-assisted syndecan-1 association with IFG1R and HER2 (Wang *et al.*, 2015); and finally, in myeloma cells, the heparanase-mediated trimming of the HS on syndecan-1 and the subsequent MMP9-mediated shedding of this syndecan, exposing a juxtamembrane site in syndecan-1 that binds VEGFR2 and VLA-4, thereby coupling VEGFR2 to the integrin (Jung *et al.*, 2016). Thus, pending on the receptor combination repertoires activated in the cells, heparanase might engage different syndecans and recruit specific cargo to exosomes.

Heparanase as exosomal cargo

Recently, several enzymes, including membrane-type 1 matrix metalloproteinase (MT1-MP), insulin-degrading enzyme (IDE), sialidase, and also heparanase, were localized on the surface of exosomes secreted by various cell types (Nawaz *et al.*, 2018; Sanderson *et al.*, 2019). For heparanase, such was noted both in myeloma (Thompson *et al.*, 2013) and in epithelial cells (Roucourt *et al.*, 2015), where most of the exosome-associated protein is present in enzyme precursor form. It is not clear what exosome component heparanase is bound to, but in epithelial cells exosomal heparanase resists the knock down of syndecans or heparan sulfate polymerase, suggesting the exosomes are not ‘heparanase-syndecan-syntenin-dependent and that binding involves yet to be identified exosomes and alternative receptors (Roucourt *et al.*, 2015). Apparently, these exosomal surface enzymes retain their activity and can degrade their

natural substrates present within extracellular spaces. Likewise, heparanase present on the exosome surface can be activated, possibly after exosome uptake, and is capable of degrading heparan sulfate embedded within an extracellular matrix (Bandari *et al.*, 2018). In that context it might also be worth reminding of the non-enzymatic function of heparanase, whereby even the catalytically dead enzyme supports mechanisms of cell migration and invasion (Gingis-Velitski *et al.*, 2004; Fux *et al.*, 2009). Exosomes have well established functions in polarized, directed cell migration (Sung *et al.*, 2015). Whether the presence of heparanase on exosomes may be pertinent in this context is not clear, but might deserve further investigation.

Conclusion and Prospects

Taken together, the above findings and considerations identify heparanase as a fundamental modulator of exosome biogenesis via the syndecan-syntenin-ALIX pathway, by cleaving and 'activating' the heparan sulfate chains of the syndecans. Thus, heparanase-enhanced tumor growth might in part be mediated by syndecan-syntenin exosomal communication in the tumor-host environment. It is interesting to note that, more recently, heparanase and syntenin have both also been implicated in processes of auto-phagocytosis. Exosomes and autophagy are linked through the endolysosomal pathway, and a strong interplay exists between both, operating as ‘partners in crime’ in the context of neurodegeneration and cancer (Xu *et al.*, 2018). Heparanase was found to reside within autophagosomes, and to promote autophagy, rendering heparanase-overexpressing cells more resistant to stress and chemotherapy. The mechanism underlying this increase in autophagy is not entirely clear, but likely involves reduced mTOR1 activity (Shteingauz *et al.*, 2015; Sanderson *et al.*, 2017). Syntenin is suppressing high levels of autophagy, while helping to maintain the protective autophagy that allows tumors stem cells to resist anoikis (Talukdar *et al.*, 2018). A possible relation between heparanase and syntenin in autophagy remains to be explored. Both might have synergic effects on signaling processes that support autophagocytosis. It might be noted that the origin of the membranes that lead to the formation of the phagophore and its elongation, to yield the double membrane that outlines the autophagosomes, remains a matter of debate, but includes the ER, ERGIC, Golgi, plasmamembrane and recycling endosomes (Mercer *et al.*, 2018). Yet, a membrane compartment with a high luminal charge of HSPG would seem improbable. Conceivably, heparanase activity helps insuring a source of such membrane. The sealing of the double membrane around the cargo to be sequestered to form an autophagosome is

topologically equivalent to membrane abscission during endosomal ILV and exosome formation, and conceivably could depend on syntenin-mediated mechanisms of recruitment. It might be noted that the ATG12-ATG3 complex involved in autophagocytosis binds and recruits ALIX (Murrow *et al*., 2015), and that ULK1 phosphorylates syntenin, modulating its non-canonical interaction with ubiquitin (Rajesh *et al*., 2011). Increasingly, attention is now also provided to non-autophagic functions of autophagy-related proteins that include secretion, trafficking of phagocytosed material and egress of viral particles (Cadwell and Debnath, 2018). Directly or indirectly, 'secretory' autophagy and exosome production might have more effectors in common than initially suspected. Possibly, some of the above considerations are also relevant for other modes of 'non-conventional' secretion; e.g., that of pro-inflammatory cytokines such as IL-1β (Dupont *et al*., 2011; Claude-Taupin *et al*., 2017). Much further work is needed, but exciting novel insight can be anticipated here.

It will be of particular interest to delineate the influence of heparanase, i.e., the heparanase-activated syndecan-syntenin-ALIX machinery, on the overall composition of exosomal cargo. Sorting of many membrane proteins into exosomes coincides with their association with tetraspanin membrane proteins (Andreu and Yáñez-Mó, 2014). Non-tetraspanin membrane proteins may piggy-back onto tetraspanin webs for their sorting into exosomes. Interestingly, with the help of syndecan, the tetraspanin CD63, which is highly enriched in exosomes, can also be recruited by syntenin (Baietti et al., 2012). The exosomal levels of CD63 are also modulated by heparanase. The levels of CD9, in contrast, are not. Sorting of tetraspanin webs at endosomes into exosomes could thus, similar to syndecans, be driven by the cytoplasmic adaptor syntenin, and the recruitment by syntenin of tetraspanin webs and syndecan clusters are thus integrated processes. All in all, a complex picture is emerging, in which both CD63 and syndecans, and possibly other membrane proteins that associate with endosomal syndecan and/or tetraspanin-enriched microdomains, are sorted into exosomes by a shared syntenin-ALIX-ESCRT machinery (Stoorvogel, 2015).

Specific exosomal cargo (Wnt11 and c-Met, respectively) has been shown to regulate crucial processes such as cancer cell motility, the onset of metastasis and premetastatic niche formation (Luga *et al*., 2012; Peinado *et al*., 2012). Intriguingly, the signalling pathways involved are strongly influenced by heparan sulfate (Hacker *et al*., 2005; Sakata *et al*., 1997), arguing for a potential pivotal role of the syndecan-syntenin-ALIX machinery and its modulators, like heparanase, in physiological processes linked to exosomes and the transfers of exosomal cargo. Of note, syntenin supports non-canonical Wnt-signalling (Egea-Jimenez *et al*., 2016) and directional cell movements in *Xenopus* and zebrafish embryos (Luyten *et al*.,

2008; Lambaerts *et al*., 2012). In zebrafish embryos, the yolk syncytial layer releases extracellular vesicles with exosome features into the blood circulation. These exosomes are released in a syntenin-dependent manner, and are captured, endocytosed and degraded by patrolling macrophages and endothelial cells, affecting the growth of the caudal vein plexus (Verweij *et al.*, 2019). Heparanase stimulates the migration of vascular endothelial cells, via protein kinase B/Akt activation (Gingis-Velitski *et al*., 2004), and is actively involved in the regulation of VEGF gene expression, mediated by activation of Src family members (Zetser *et al*., 2006). Recently, c-Src, phosphorylating both syntenin and the ICD of syndecan, was identified as a cytosolic activator of the syntenin-exosome pathway, and syntenin-exosomes as a requirement for non-cell autonomous effects of c-Src on vascular endothelial cell motility (Imjeti *et al*., 2017). Conceivably, heparanase and c-Src may thus sustain a positive feedback loop in exosomal communication. Yet, in essence all this remains to be explored. If such proves to be the case, syntenin, heparanase and c-Src, all often upregulated in cancer, represent interesting targets for modulating exosome effects in cancer therapies.

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References

Andreu, Z., Yáñez-Mó, M. (2014) Tetraspanins in extracellular vesicle formation and function. *Front. Immunol.* 5, 442

Babst, M. (2011) MVB vesicle formation: ESCRT-dependent, ESCRT-independent and everything in between. *Curr. Opin. Cell Biol.* 23, 452–457

Bandari, S.K., Purushothaman, A., Ramani, V.C., Brinkley, G.J., Chandrashekar, D.S., Varambally, S., Mobley, .JA., Zhang, Y., Brown, E.E., Vlodavsky, I., Sanderson, R.D. (2018) Chemotherapy induces secretion of exosomes loaded with heparanase that degrades extracellular matrix and impacts tumor and host cell behavior. *Matrix Biol.* 65, 104-118

Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E., Zimmermann, P., David, G. (2012) Syndecan–syntenin–ALIX regulates the biogenesis of exosomes. *Nat. Cell Biol.* 14, 677–685

Beauvais, D.M., Jung, O., Yang, Y., Sanderson, R.D., Rapraeger, A.C. (2016) Syndecan-1 (CD138) Suppresses Apoptosis in Multiple Myeloma by Activating IGF1 Receptor: Prevention by SynstatinIGF1R Inhibits Tumor Growth. 1. *Cancer Res.* 76, 4981-93

Beauvais, D.M., Rapraeger, A.C. (2010) Syndecan-1 couples the insulin-like growth factor-1 receptor to inside-out integrin activation. *J. Cell Sci.* 123, 3796-807

Buschow, S.I., Nolte-'t Hoen, E.N., van Niel, G., Pols, M.S., ten Broeke, T., Lauwen, M., Ossendorp, F., Melief, C.J., Raposo, G., Wubbolts, R., Wauben, M.H., Stoorvogel, W. (2009 MHC II in dendritic cells is targeted to lysosomes or T cell-induced exosomes via distinct multivesicular body pathways. *Traffic* 10, 1528-1542

Cadwell, K., Debnath, J. (2018) Beyond self-eating: The control of nonautophagic functions and signaling pathways by autophagy-related proteins. *J. Cell Biol.* 217, 813-822

Christianson, H.C., Belting, M. (2014) Heparan sulfate proteoglycan as a cell-surface endocytosis receptor. *Matrix Biol.* 35, 51–55

Christianson, H.C., Svensson, K.J., van Kuppevelt, T.H., Li, J.P., Belting, M. (2013) Cancer cell exosomes depend on cell-surface heparan sulfate proteoglycans for their internalization and functional activity. *Proc. Natl. Acad. Sci. USA* 110, 17380–17385

Claude-Taupin, A., Jia, J., Mudd, M., Deretic, V. (2017) Autophagy's secret life: secretion instead of degradation. *Essays Biochem.* 61, 637-647

Colombo, M., Moita, C., van Niel, G., Kowal, J., Vigneron, J., Benaroch, P., Manel, N., Moita, L.F., Théry, C., Raposo, G. (2013) Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci.* 126, 5553-65

Colombo, M., Raposo, G., Thery, C. (2014) Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol.* 30, 255–289

David, G., Zimmermann, P. (2015) Heparanase tailors syndecan for exosome production. *Mol Cell Oncol.* 3, e1047556

Dupont, N., Jiang, S., Pilli, M., Ornatowski, W., Bhattacharya, D., Deretic, V. (2011) Autophagy-based unconventional secretory pathway for extracellular delivery of IL-1β. *EMBO J.* 30, 4701-11

Edovitsky E, Elkin M, Zcharia E, Peretz T, Vlodavsky I. (2004) Heparanase gene silencing, tumor invasiveness, angiogenesis, and metastasis. *J. Natl. Cancer Inst.* 96, 1219–1230

Egea-Jimenez, A.L., Gallardo, R., Garcia-Pino, A., Ivarsson, Y., Wawrzyniak, A.M., Kashyap, R., Loris, R., Schymkowitz, J., Rousseau, F., Zimmermann P. (2016) Frizzled 7 and PIP2 binding by syntenin PDZ2 domain supports Frizzled 7 trafficking and signalling. *Nat. Commun*. 2016 7, 12101

Egea-Jimenez, A.L., Zimmermann, P. (2018) Phospholipase D and phosphatidic acid in the biogenesis and cargo loading of extracellular vesicles. *J Lipid Res.* 59, 1554-1560

Fares, J., Kashyap, R., Zimmermann, P. (2017) Syntenin: Key player in cancer exosome biogenesis and uptake? *Cell Adh. Migr.* 11,124-126

Feng, D., Zhao, W.L., Ye, Y.Y., Bai, X.C., Liu, R.Q., Chang, L.F., Zhou, Q., Sui, S.F. (2010) Cellular internalization of exosomes occurs through phagocytosis. *Traffic* 11:675-87.

Fevrier, B., Vilette, D., Archer, F., Loew, D., Faigle, W., Vidal, M., Laude, H., Raposo, G. (2004) Cells release prions in association with exosomes. *Proc. Natl. Acad. Sci. USA* 101, 9683–9688

French, K.C., Antonyak, M.A., Cerione, R.A. (2017) Extracellular vesicle docking at the cellular port: Extracellular vesicle binding and uptake. *Semin. Cell Dev. Biol.* 67, 48-55

Friand, V., David, G., Zimmermann, P. (2015) Syntenin and syndecan in the biogenesis of exosomes. *Biol. Cell.* 107, 331-41

Fujii, K., Hurley, J.H., Freed, E.O. (2007) Beyond Tsg101: the role of Alix in 'ESCRTing' HIV-1. *Nat. Rev. Microbiol.* 5, 912–916

Fuster, M.M., Esko, J.D. (2005) The sweet and sour of cancer: glycans as novel therapeutic targets. *Nat. Rev. Cancer* 5, 526–542

Fux, L., Feibish, N., Cohen-Kaplan, V., Gingis-Velitski, S., Feld, S., Geffen, C., Vlodavsky, I., Ilan, N. (2009) Structure-function approach identifies a COOH-terminal domain that mediates heparanase signaling. *Cancer Res.* 69, 1758–1767

Garrido-Urbani, S., Garg, P., Ghossoub, R., Arnold, R., Lembo, F., Sundell, G.N., Kim, P.M., Lopez, M., Zimmermann, P., Sidhu, S.S., Ivarsson, Y. (2016) Proteomic peptide phage display uncovers novel interactions of the PDZ1-2 supramodule of syntenin. *FEBS Lett.* 590, 3-12

Gingis-Velitski, S., Zetser, A., Flugelman, M.Y., Vlodavsky, I., Ilan, N. (2004) Heparanase induces endothelial cell migration via protein kinase B/Akt activation. *J. Biol. Chem.* 279, 23536–23541

Gingis-Velitski, S., Zetser, A., Kaplan, V., Ben-Zaken, O., Cohen, E., Levy-Adam, F., Bashenko, Y., Flugelman, M.Y., Vlodavsky, I., Ilan, N. (2004) Heparanase uptake is mediated by cell membrane heparan sulfate proteoglycans. *J. Biol. Chem*. 279, 44084-92

Ghossoub, R., Lembo, F., Rubio, A., Gaillard, C.B., Bouchet, J., Vitale, N., Slavik, J., Machala, M., Zimmermann, P. (2014) Syntenin–ALIX exosome biogenesis and budding into multivesicular bodies are controlled by ARF6 and PLD2. *Nat. Commun.* 5, 3477

Gould, S.J., Booth, A.M., Hildreth, J.E. (2003) The Trojan exosome hypothesis. *Proc. Natl. Acad. Sci. U S A.* 100, 10592-7

Gräßel, L., Fast, L.A., Scheffer, K.D., Boukhallouk, F., Spoden, G.A., Tenzer, S., Boller, K., Bago, R., Rajesh, S., Overduin, M., Berditchevski, F., Florin, L. (2016) The CD63-Syntenin-1 Complex Controls Post-Endocytic Trafficking of Oncogenic Human Papillomaviruses. *Sci. Rep.* 6:32337

Grootjans, J.J., Reekmans, G., Ceulemans, H., David, G. (2000) Syntenin–syndecan binding requires syndecan–synteny and the co-operation of both PDZ domains of syntenin. *J. Biol. Chem.* 275, 19933–19941

Grootjans, J.J., Zimmermann, P., Reekmans, G., Smets, A., Degeest, G., Durr, J., David, G. (1997) Syntenin, a PDZ protein that binds syndecan cytoplasmic domains. *Proc. Natl. Acad. Sci. USA* 94, 13683–13688

Hacker, U., Nybakken, K., Perrimon, N. (2005) Heparan sulphate proteoglycans: the sweet side of development. *Nat. Rev. Mol. Cell Biol.* 6, 530–541

Hadigal, S.R., Agelidis, A.M., Karasneh, G.A., Antoine, T.E., Yakoub, A.M., Ramani, V.C., Djalilian, A.R., Sanderson, R.D., Shukla, D. (2015) Heparanase is a host enzyme required for herpes simplex virus-1 release from cells. *Nat Commun*. 6, 6985

Hanson, P.I., Cashikar, A. (2012) Multivesicular body morphogenesis. *Annu. Rev. Cell Dev. Biol.* 28, 337–362

Henne, W.M., Buchkovich, N.J., Emr, S.D. (2011) The ESCRT pathway. *Dev. Cell* 21, 77–91

Hergenreider, E., Heydt, S., Treguer, K., Boettger, T., Horrevoets, A.J., Zeiher, A.M., Scheffer, M.P., Frangakis, A.S., Yin, X., Mayr, M., Braun, T., Urbich, C., Boon, R.A., Dimmeler, S. (2012) Atheroprotective communication between endothelial cells and smooth muscle cells through miRNAs. *Nat. Cell Biol.* 14, 249–256

Hurley, J.H. (2015) ESCRTs are everywhere. *EMBO J.* 34, 2398-407

Hurley, J.H., Cada, A.K. (2018) Inside job: how the ESCRTs release HIV-1 from infected cells. *Biochem. Soc. Trans.* 46, 1029-1036

Hurley, J.H., Hanson, P.I. (2010) Membrane budding and scission by the ESCRT machinery: it's all in the neck. *Nat. Rev. Mol. Cell Biol.* 11, 556–566

Hurley, J.H., Odorizzi, G. (2012) Get on the exosome bus with ALIX. *Nat Cell Biol.* 14, 654–655

Imjeti, N.S., Menck, K., Egea-Jimenez, A.L., Lecointre, C., Lembo, F., Bouguenina, H., Badache, A., Ghossoub, R., David, G., Roche, S., Zimmermann, P. (2017) Syntenin mediates SRC function in exosomal cell-to-cell communication. *Proc. Natl. Acad. Sci. U S A.* 114, 12495-12500

Jung, O., Trapp-Stamborski, V., Purushothaman, A., Jin, H., Wang, H., Sanderson, R.D., Rapraeger, A.C. (2016) Heparanase-induced shedding of syndecan-1/CD138 in myeloma and endothelial cells activates VEGFR2 and an invasive phenotype: prevention by novel synstatins. *Oncogenesis.* 5:e202.

Kato, M., Wang, H., Kainulainen, V., Fitzgerald, M.L., Ledbetter, S., Ornitz, D.M., Bernfield, M. (1998) Physiological degradation converts the soluble syndecan-1 ectodomain from an inhibitor to a potent activator of FGF-2. *Nat. Med.* 4, 691–697

Kowal, J., Tkach, M., Thery, C. (2014) Biogenesis and secretion of exosomes. *Curr. Opin. Cell Biol.* 29, 116–125

Kowal, J., Arras, G., Colombo, M., Jouve, M., Morath, J.P., Primdal-Bengtson, B., Dingli, F., Loew, D., Tkach, M., Théry, C. (2016) Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc. Natl. Acad. Sci. U S A.* 113, E968-77

Lambaerts, K., Van Dyck, S., Mortier, E., Ivarsson, Y., Degeest, G., Luyten, A., Vermeiren, E., Peers, B., David, G., Zimmermann, P. (2012) Syntenin, a syndecan adaptor and an Arf6 phosphatidylinositol 4,5-bisphosphate effector, is essential for epiboly and gastrulation cell movements in zebrafish. *J. Cell Sci.* 125, 1129–1140

Lambaerts, K., Wilcox-Adelman, S.A., Zimmermann, P. (2009) The signaling mechanisms of syndecan heparan sulfate proteoglycans. *Curr. Opin. Cell Biol.* 21, 662–669

Latysheva, N., Muratov, G., Rajesh, S., Padgett, M., Hotchin, N.A., Overduin, M., Berditchevski, F. (2006) Syntenin-1 is a new component of tetraspanin-enriched microdomains: mechanisms and consequences of the interaction of syntenin-1 with CD63. *Mol. Cell Biol.* 26, 7707-18

Laulagnier, K., Grand, D., Dujardin, A., Hamdi, S., Vincent-Schneider, H., Lankar, D., Salles, J.P., Bonnerot, C., Perret, B., Record, M. (2004) PLD2 is enriched on exosomes and its activity is correlated to the release of exosomes. *FEBS Lett.* 572, 11–14

Lindahl, U., Kjellen, L. (2013) Pathophysiology of heparan sulphate: many diseases, few drugs. *J. Intern. Med.* 273, 555–571

Luga, V., Zhang, L., Viloria-Petit, A.M., Ogunjimi, A.A., Inanlou, M.R., Chiu, E., Buchanan, M., Hosein, A.N., Basik, M., Wrana, J.L. (2012) Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151, 1542-56

Luyten, A., Mortier, E., Van Campenhout, C., Taelman, V., Degeest, G., Wuytens, G., Lambaerts, K., David, G., Bellefroid, E.J., Zimmermann, P. (2008) The postsynaptic density 95/disc-large/zona occludens protein syntenin directly interacts with frizzled 7 and supports noncanonical Wnt signaling. *Mol. Biol. Cell* 19, 1594–1604

Ma, P., Beck, S.L., Raab, R.W., McKown, R.L., Coffman, G.L., Utani, A., Chirico, W.J., Rapraeger, A.C., Laurie, G. W. (2006) Heparanase deglycanation of syndecan-1 is required for binding of the epithelial-restricted prosecretory mitogen lacritin. *J. Cell Biol.* 174, 1097-106.

Mali, M., Elenius, K., Miettinen, H.M., Jalkanen, M. (1993) Inhibition of basic fibroblast growth factor-induced growth promotion by overexpression of syndecan-1. *J. Biol. Chem.* 268, 24215–24222

Mathieu, M., Martin-Jaular, L., Lavieu, G., Théry, C. (2019) Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat. Cell Biol*. 21, 9-17

Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Fauré, J., Blanc, N.S., Matile, S., Dubochet, J., Sadoul, R., Parton, R.G., Vilbois, F., Gruenberg, J. (2004) Role of LBPA and Alix in multivesicular liposome formation and endosome organization. *Science* 303, 531-4.

Mercer, T.J., Gubas, A., Tooze, S.A. (2018) A molecular perspective of mammalian autophagosome biogenesis. *J. Biol. Chem.* 293, 5386-5395

Morita, E., Sandrin, V., McCullough, J., Katsuyama, A., Baci Hamilton, I., Sundquist, W.I. (2011) ESCRT-III protein requirements for HIV-1 budding. *Cell Host Microbe* 9, 235–242

Mulcahy, L.A., Pink, R.C., Carter, D.R. (2014) Routes and mechanisms of extracellular vesicle uptake. *J. Extracell. Vesicles* 3, 24641

Murrow, L., Malhotra, R., Debnath, J. (2015) ATG12-ATG3 interacts with Alix to promote basal autophagic flux and late endosome function. *Nat. Cell Biol.* 17, 300-10

Nakase, I., Kobayashi, N.B., Takatani-Nakase, T., Yoshida, T. (2015) Active macropinocytosis induction by stimulation of epidermal growth factor receptor and oncogenic Ras expression potentiates cellular uptake efficacy of exosomes. *Sci. Rep.* 5, 10300

Nawaz, M., Shah, N., Zanetti, B.R., Maugeri, M., Silvestre, R.N., Fatima, F., Neder, L., Valadi, H. (2018) Extracellular Vesicles and Matrix Remodeling Enzymes: The Emerging Roles in Extracellular Matrix Remodeling, Progression of Diseases and Tissue Repair. *Cells* 7, 167

Nolte-'t Hoen, E., Cremer, T., Gallo, R.C., Margolis, L.B. (2016) Extracellular vesicles and viruses: Are they close relatives? *Proc. Natl. Acad. Sci. U S A.* 113, 9155-61

Peinado, H., Aleckovic, M., Lavotshkin, S., Matei, I., Costa-Silva, B., Moreno-Bueno, G., Hergueta-Redondo, M., Williams, C., Garcia-Santos, G., Ghajar, C., Nitadori-Hoshino, A., Hoffman, C., Badal, K., Garcia, B.A., Callahan, M.K., Yuan, J., Martins, V.R., Skog, J., Kaplan, R.N., Brady, M.S., Wolchok, J.D., Chapman, P.B., Kang, Y., Bromberg, J., Lyden, D. (2012) Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat. Med.* 18, 883–891

Purushothaman, A., Bandari, S.K., Liu, J., Mobley, J.A., Brown, E.E., Sanderson, R.D. (2016) Fibronectin on the Surface of Myeloma Cell-derived Exosomes Mediates Exosome-Cell Interactions. *J. Biol. Chem.* 291, 1652-63

Raiborg, C., Bremnes, B., Mehlum, A., Gillooly, D.J., D'Arrigo, A., Stang, E., Stenmark, H. (2001) FYVE and coiled-coil domains determine the specific localisation of Hrs to early endosomes. *J. Cell Sci.* 114, 2255-63.

Raiborg, C., Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* 458, 445–452

Rajendran, L., Honsho, M., Zahn, T.R., Keller, P., Geiger, K.D., Verkade, P., Simons, K. (2006) Alzheimer's disease beta-amyloid peptides are released in association with exosomes. *Proc. Natl. Acad. Sci. USA* 103, 11172–11177

Rajesh, S., Bago, R., Odintsova, E., Muratov, G., Baldwin, G., Sridhar, P., Overduin, M., Berditchevski, F. (2011) Binding to syntenin-1 protein defines a new mode of ubiquitin-based interactions regulated by phosphorylation. *J. Biol. Chem.* 286, 39606–39614

Raposo, G., Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383

Reiland, J., Kempf, D., Roy, M., Denkins, Y., Marchetti, D. (2006) FGF2 binding, signaling, and angiogenesis are modulated by heparanase in metastatic melanoma cells. *Neoplasia* 8, 596–606

Roucourt, B., Meeussen, S., Bao, J., Zimmermann, P., David, G. (2015) Heparanase activates the syndecan–syntenin–ALIX exosome pathway. *Cell Res.* 25, 412–428

Sakata, H., Stahl, S.J., Taylor, W.G., Rosenberg, J.M., Sakaguchi, K., Wingfield, P.T., Rubin, J.S. (1997) Heparin binding and oligomerization of hepatocyte growth factor/scatter factor isoforms. Heparan sulfate glycosaminoglycan requirement for Met binding and signaling. *J. Biol. Chem.* 272, 9457–9463

Sanderson, R.D., Bandari, S.K., Vlodavsky, I. (2019) Proteases and glycosidases on the surface of exosomes: Newly discovered mechanisms for extracellular remodeling. *Matrix Biol.* 75-76, 160-169

Sanderson, R.D., Elkin, M., Rapraeger, A.C., Ilan, N., Vlodavsky, I. (2017) Heparanase regulation of cancer, autophagy and inflammation: new mechanisms and targets for therapy. *FEBS J.* 284, 42-55.

Sarrazin, S., Lamanna, W.C., Esko, J.D. (2011) Heparan sulfate proteoglycans. *Cold Spring Harb. Perspect. Biol.* 3, 7.

Schiro, A., Wilkinson, F.L., Weston, R., Smyth, J.V., Serracino-Inglott, F., Alexander, M.Y. (2014) Endothelial microparticles as conveyors of information in atherosclerotic disease. *Atherosclerosis* 234, 295–302

Schlessinger, J., Plotnikov, A.N., Ibrahimi, O.A., Eliseenkova, A.V., Yeh, B.K., Yayon, A., Linhardt, R.J., Mohammadi, M. (2000) Crystal structure of a ternary FGF-FGFR-heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* 6, 743-50

Schneider, A., Simons, M. (2013) Exosomes: vesicular carriers for intercellular communication in neurodegenerative disorders. *Cell Tissue Res.* 352, 33–47

Schöneberg, J., Lee, I.H., Iwasa, J.H., Hurley, J.H. (2017) Reverse-topology membrane scission by the ESCRT proteins. *Nat. Rev. Mol. Cell Biol.* 18, 5-17.

Shteingauz, A., Boyango, I., Naroditsky, I., Hammond, E., Gruber, M., Doweck, I., Ilan, N., Vlodavsky, I. (2015) Heparanase Enhances Tumor Growth and Chemoresistance by Promoting Autophagy. *Cancer Res.* 75:3946-57

Shukla, D., Liu, J., Blaiklock, P., Shworak, N.W., Bai, X., Esko, J.D., Cohen, G.H., Eisenberg, R.J., Rosenberg, R.D., Spear, P.G. (1999) A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell* 99, 13-22

Simons, M., Raposo, G. (2009) Exosomes – vesicular carriers for intercellular communication. *Curr. Opin. Cell Biol.* 21, 575–581

Skog, J., Würdinger, T., van Rijn, S., Meijer, D.H., Gainche, L., Sena-Esteves, M., Curry, W.T. Jr, Carter, B.S., Krichevsky, A.M., Breakefield, X.O. (2008) Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat. Cell Biol.* 10, 1470–1476

Stoorvogel, W. (2015) Resolving sorting mechanisms into exosomes. *Cell Res.* 25, 531-2

Strack, B., Calistri, A., Craig, S., Popova, E., Gottlinger, H.G. (2003) AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding. *Cell* 114, 689–699

Sung, B.H., Ketova, T., Hoshino, D., Zijlstra, A., Weaver, A.M. (2015) Directional cell movement through tissues is controlled by exosome secretion. *Nat. Commun.* 6, 7164

Talukdar, S., Pradhan, A.K., Bhoopathi, P., Shen, X.N., August, L.A., Windle, J.J., Sarkar, D., Furnari, F.B., Cavenee, W.K., Das, S.K., Emdad, L., Fisher, P.B. (2018) MDA-9/Syntenin regulates protective autophagy in anoikis-resistant glioma stem cells. *Proc. Natl. Acad. Sci. U S A.* 115, 5768-5773

Thakkar, N., Yadavalli, T., Jaishankar, D., Shukla, D. (2017) Emerging Roles of Heparanase in Viral Pathogenesis. *Pathogens* 6, 43

Thery, C., Duban, L., Segura, E., Veron, P., Lantz, O., Amigorena, S. (2002) Indirect activation of naive CD4+ T cells by dendritic cell-derived exosomes. *Nat. Immunol.* 3, 1156–1162

Thompson, C.A., Purushothaman, A., Ramani, V.C., Vlodavsky, I., Sanderson, R.D. (2013) Heparanase regulates secretion, composition, and function of tumor cell-derived exosomes. *J. Biol. Chem.* 288, 10093–10099

Tkach, M., Théry, C. (2016) Communication by Extracellular Vesicles: Where We Are and Where We Need to Go. *Cell* 164, 1226-1232

Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brugger, B., Simons, M. (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 319, 1244–1247

Valadi, H., Ekstrom, K., Bossios, A., Sjostrand, M., Lee, J.J., Lotvall, J.O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659

van Niel, G., D'Angelo, G., Raposo, G. (2018) Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* 19, 213-228

Verweij, F.J., Revenu, C., Arras, G., Dingli, F., Loew, D., Pegtel, D.M., Follain, G., Allio, G., Goetz, J.G., Zimmermann, P., Herbomel, P., Del Bene, F., Raposo, G., van Niel, G. (2019) Live Tracking of Inter-organ Communication by Endogenous Exosomes In Vivo. *Dev. Cell* 48, 573-589

Vlodavsky, I., Friedmann, Y., Elkin, M., Aingorn, H., Atzmon, R., Ishai-Michaeli, R., Bitan, M., Pappo, O., Peretz, T., Michal, I., Spector, L., Pecker, I. (1999) Mammalian heparanase: gene cloning, expression and function in tumor progression and metastasis. *Nat. Med.* 5, 793-802

Vreys, V., Delande, N., Zhang, Z., Coomans, C., Roebroek, A., Durr, J., David, G. (2005) Cellular uptake of mammalian heparanase precursor involves low density lipoprotein receptor-related proteins, mannose 6-phosphate receptors, and heparan sulfate proteoglycans. *J. Biol. Chem.* 280, 33141–33148

Wang, H., Jin, H., Rapraeger, A.C. (2015) Syndecan-1 and Syndecan-4 Capture Epidermal Growth Factor Receptor Family Members and the α3β1 Integrin via Binding Sites in Their Ectodomains: Novel Synstatins Prevent Kinase Capture and Inhibit α6β4-Integrin-dependent Epithelial Cell Motility. *J. Biol. Chem.* 290:26103-13

Wang, J., Wang, N., Xie, J., Walton, S.C., McKown, R.L., Raab, R.W., Ma, P., Beck, S.L., Coffman, G.L., Hussaini, I.M., Laurie, G.W. (2006) Restricted epithelial proliferation by lacritin via PKCalpha-dependent NFAT and mTOR pathways. *J. Cell Biol.* 174, 689-700

Wang, N., Zimmerman, K., Raab, R.W., McKown, R.L., Hutnik, C.M., Talla, V., Tyler, M.F. 4th, Lee, J.K., Laurie, G.W. (2013) Lacritin rescues stressed epithelia via rapid forkhead box O3 (FOXO3)-associated autophagy that restores metabolism. *J. Biol. Chem.* 288, 18146-61

Wills, J.W., Cameron, C.E., Wilson, C.B., Xiang, Y., Bennett, R.P., Leis, J. (1994) An assembly domain of the Rous sarcoma virus Gag protein required late in budding. *J. Virol.* 68, 6605–6618

Whiteford, J.R., Xian, X., Chaussade, C., Vanhaesebroeck, B., Nourshargh, S., Couchman, J.R. (2011) Syndecan-2 is a novel ligand for the protein tyrosine phosphatase receptor CD148. *Mol. Biol. Cell* 22, 3609-24

Wollert, T., Hurley, J.H. (2010) Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. *Nature* 464, 864–869

Wurdinger, T., Gatson, N.N., Balaj, L., Kaur, B., Breakefield, X.O., Pegtel, D.M. (2012) Extracellular vesicles and their convergence with viral pathways. *Adv. Virol.* 2012, 767694

Xu, J., Camfield, R., Gorski, S.M. (2018) The interplay between exosomes and autophagy - partners in crime. *J. Cell Sci.* 131,215210

Yanagishita, M., Hascall, V.C. (1992) Cell surface heparan sulfate proteoglycans. *J. Biol. Chem.* 267, 9451–9454

Yang, J.M., Gould, S.J. (2013) The cis-acting signals that target proteins to exosomes and microvesicles. *Biochem. Soc. Trans.* 41, 277–282

Yang, Y., Macleod, V., Miao, H.Q., Theus, A., Zhan, F., Shaughnessy, J.D., Jr., Sawyer, J., Li, J.P., Zcharia, E., Vlodavsky, I., Sanderson, R.D. (2007) Heparanase enhances syndecan-1 shedding: a novel mechanism for stimulation of tumor growth and metastasis. *J. Biol. Chem.* 282, 13326–13333

Zcharia, E., Jia, J., Zhang, X., Baraz, L., Lindahl, U., Peretz, T., Vlodavsky, I., Li, J.P. (2009) Newly generated heparanase knock-out mice unravel co-regulation of heparanase and matrix metalloproteinases. *PLoS One* 4, e5181

Zetser, A., Bashenko, Y., Edovitsky, E., Levy-Adam, F., Vlodavsky, I., Ilan, N. (2006) Heparanase induces vascular endothelial growth factor expression: correlation with p38 phosphorylation levels and Src activation. *Cancer Res.* 66, 1455–1463

Zetser, A., Levy-Adam, F., Kaplan, V., Gingis-Velitski, S., Bashenko, Y., Schubert, S., Flugelman, M.Y., Vlodavsky, I., Ilan, N. (2004) Processing and activation of latent heparanase occurs in lysosomes. *J. Cell Sci.* 117, 2249–2258

Zhang, Y., Wang, N., Raab, R.W., McKown, R.L., Irwin, J.A., Kwon, I., van Kuppevelt, T.H., Laurie, G.W. (2013) Targeting of heparanase-modified syndecan-1 by prosecretory mitogen lacritin requires conserved core GAGAL plus heparan and chondroitin sulfate as a novel hybrid binding site that enhances selectivity. *J. Biol. Chem.* 288, 12090-101

Zhang, Z., Coomans, C., David, G. (2001) Membrane heparan sulfate proteoglycan-supported FGF2–FGFR1 signaling: evidence in support of the "cooperative end structures" model. *J. Biol. Chem.* 276, 41921–41929

Zimmermann, P., Meerschaert, K., Reekmans, G., Leenaerts, I., Small, J.V., Vandekerckhove, J., David, G., Gettemans, J. (2002) PIP(2)–PDZ domain binding controls the association of syntenin with the plasma membrane. *Mol. Cell* 9, 1215–1225

Zimmermann, P., Tomatis, D., Rosas, M., Grootjans, J., Leenaerts, I., Degeest, G., Reekmans, G., Coomans, C., David, G. (2001) Characterization of syntenin, a syndecan-binding PDZ protein, as a component of cell adhesion sites and microfilaments. *Mol. Biol. Cell* 12, 339–350

Zimmermann, P., Zhang, Z., Degeest, G., Mortier, E., Leenaerts, I., Coomans, C., Schulz, J., N'Kuli, F., Courtoy, P.J., David, G. (2005) Syndecan recycling [corrected] is controlled by syntenin–PIP2 interaction and Arf6. *Dev. Cell* 9, 377–388