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Organizing polarized delivery of exosomes at synapses

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Abstract

Exosomes are extracellular vesicles that transport different molecules between cells. They are formed and stored inside multivesicular bodies (MVB) until they are released to the extracellular environment. MVB fuse along the plasma membrane, driving non-polarized secretion of exosomes. However, polarized signalling can potentially direct MVBs to a specific point in the plasma membrane to mediate a focal delivery of exosomes. MVB polarization occurs across a broad set of cellular situations, e.g. in immune and neuronal synapses, cell migration and in epithelial sheets. In this review, we summarize the current understanding regarding polarized MVB docking and the specification of secretory sites at the plasma membrane. The current view is that MVB positioning and subsequent exosome delivery requires a polarizing, cytoskeletal dependent- trafficking mechanism. In this context, we propose scenarios in which biochemical and mechanical signals could drive the polarized delivery of exosomes in highly polarized cells, such as lymphocytes, neurons and epithelia.

1 Introduction

Cells release different types of vesicles to the extracellular environment (1). Extracellular vesicles (EV) are classified according to their subcellular origin: shedding vesicles are formed by direct budding of the plasma membrane, whereas exosomes are formed inside late endosomes (or multivesicular bodies, MVBs) by budding of the limiting membrane and are released into the extracellular medium upon MVB fusion with the plasma membrane. The subcellular origin of the EVs determines the dynamics and kinetics of secretion and, although it is not yet known, it is feasible that their origin could also affect the function and the fate of the EVs.

Exosomes transport proteins, such as receptors and intracellular signaling molecules, lipids, a variety of RNA and even DNA between cells. They have emerged as important vehicles for cell-to-cell communication (2). Due to their role in cellular communication, exosomes have been involved in a variety of physiological processes, including fetal development (3),

neuronal communication (4), immunological responses (5), and tissue repair (6). Recently, exosomes secreted by male reproductive glands have been reported to be able to reprogram female behavior in *Drosophila melanogaster* (7).

Exosomes are particularly important in structures for defining close contacts between cells (2), e.g. synapses, where they act in an autocrine or paracrine manner. They can also act at a distance, since they are found in all body fluids (8). Exosomes are present in blood and other fluids and do not degrade in response to shearing forces, making them excellent vehicles to transfer shear-sensitive molecules, e.g. miRNA, to distant tissues. Hence, cells can use them to shuttle sophisticated gene expression-modulating signals at long distances.

Exosomes were originally described in 1984 believed to function to rid the cell of unwanted proteins (9). Exosomes are coordinated within the autophagy/lysosome recycling system for the maintenance of cellular proteostasis (10). They have come into the spotlight only recently, particularly after the finding that they contain mRNA and microRNA that is transferred between cells (11, 12). Early studies revealed that EVs secreted by embryonic stem cells or tumor cells contain mRNA. These transcripts can be delivered to target cells, where they are translated into functional proteins (11, 12). The RNA composition of EVs is not a mere reflection of the whole RNA produced by the cell; Some microRNAs are highly enriched in EV, whereas others are excluded (13, 14), indicating the existence of specific sorting mechanisms (14). RNA deep sequencing studies have demonstrated that, besides miRNAs, EVs contain other small non-coding RNA species such as fragments of structural RNAs and repeat sequences (11, 13). The exosomal protein repertoire is also a consequence of complex mechanisms that control their loading into exosomes. Exosomes are enriched in tetraspanins and associated membrane proteins, such as integrins, cytoskeletal proteins, ESCRT-related proteins and heat-shock proteins (15).

Unlike shedding vesicles, exosomes are “stored” inside late endosomes and their secretion can be spatially and temporally directed. In this review, we offer a perspective of the current state of the art regarding the transport of MVB to a specific membrane location, and drive the delivery of exosomes in a polarized manner. We discuss the evidence pertaining the involvement of cellular cytoskeletons, the small GTPases and the phospholipid signaling circuits to drive the polarization of MVBs toward a specific point of the plasma membrane.

2 Moving in: MVB maturation and movement to the cell center

Contrary to other types of extracellular vesicles, exosomes have an endocytic origin and are formed as intraluminal vesicles (ILVs) by inward budding of the limiting membrane of endosomes. While early endosomes mature into late endosomes, they accumulate ILV into their lumen. Different sorting mechanisms exist to sequester molecules for ILV which include recognition of post-translational modifications, including ubiquitination, SUMOylation, phosphorylation and glycosylation (16). The best characterized MVB sorting mechanism is the ESCRT machinery. ESCRT complexes include ESCRT-0, -I, -II and -III. ESCRT-0 mediates the targeting of ubiquitinated transmembrane proteins. ESCRT-0 is recruited to MVB as one of its subunits, Vps27, contains a FYVE domain that specifically binds PtdIns(3)P (17), which is produced exclusively in MVB by endosomal class II

phosphatidylinositol 3-kinase (18). ESCRT-I forms a complex with ESCRT-II that mediates vesicle budding, while ESCRT-III contributes to membrane curvature and controls vesicle abscission. Vps4 is an AAA ATPase (ATPase Associated with a variety of cellular Activities) that is believed to provide the energy to drive vesicle abscission in an ATP-dependent manner (19). Depletion of components of all four ESCRT complexes does not abolish MVB formation in mammalian cells (20). ESCRT independent mechanism of ILV formation depending on lipids and tetraspanins has been also reported (15).

Inside a single MVB, different ILV subpopulations can be distinguished by the formation mechanisms and size. In HeLa cells, EGF stimulation promotes the formation of large ESCRT-dependent ILVs, whereas depletion of the ESCRT-0 component, Hrs, promotes the formation of a uniformly-sized population of small ILVs, which requires the tetraspanin CD63 to form (21). Silencing of specific molecules can block the production of certain types of EVs. For example, silencing syndecan or syntenin also blocks the release of exosomes bearing CD63 and Hsp70 without altering the release of flotillin-positive vesicles (22). Biogenesis could be important to polarized secretion of exosomes, since secretory MVBs are likely to be different from those fated for fusion with lysosomes for degradation. In B cell lines, cholesterol-positive and -negative MVB coexist, and cholesterol-positive MVB preferentially fuse with the plasma membrane for exosome release, whereas LBPA vesicles are preferentially targeted to degradation (23, 24). Interestingly, epithelial cells can secrete exosomes with a different protein composition from the apical and basolateral surfaces (25).

During maturation, endosomes are transported along microtubules towards the center of the cell. This movement is propelled by dynein/dynactin molecular motors under the control of the small GTPase Rab5 (26). On the mature endosome (MVB), Rab5 has been replaced by Rab7; the Rab7 effector RILP interacts with dynein/dynactin motors complex controlling microtubule minus end transport of MVB (27). Dynein recruitment to Rab7/RILP is controlled by the cholesterol sensor ORP1L. Motor proteins, besides acting on endosome trafficking, could also dictate their morphology and cargo sorting (28). Interestingly, the microtubule-binding protein Hook has been involved in both maturation of MVB acting as a negative regulator of endosome maturation, and preventing MVB fusion with the degradative autophagy/lysosome pathway (29). Under fed conditions, Hook anchors MVBs to microtubules, impairing their fusion with autophagosome/lysosome. Autophagy induction by starvation removes Hook from MVBs by Rab11 allowing the fusion of MVB with autophagosomes (30).

3 Moving out: MVB transport to the plasma membrane, fusion and exosome release

In this section, we discuss the mechanisms by which MVB are targeted to secretory locations in the plasma membrane, including cytoplasmic transport, and the molecular events that mediate fusion with the plasma membrane and exosome secretion.

Although little information is available regarding the translocation of MVB to the plasma membrane, it seems that the transport of MVB to the periphery requires the concerted action of cytoskeleton and signalling components such as small GTPases and lipid second-

messengers. This has been best characterized in models of viral infection, e.g. dwarf rice virus, in insect cells (31). In these, actomyosin contractility regulates the intracellular positioning of virus-containing MVB. Likewise, MVB containing adenylyl cyclase are polarized to the trailing edge of migrating cells and secreted in a rearward fashion to leave a “bread crumb” trail that other cells use to follow the leader cells (32). This process requires intact actin and tubulin cytoskeletons. This process requires an intact actin and tubulin cytoskeletons. A possible mechanistic connection is the ability of K63-linked ubiquitin chains to recruit actin nucleators that drive actin polymerization (reviewed in (33)).

Regarding the specific molecules involved in these processes, several molecular players stand out. For example, Kif2c is a neuron-restricted, MVB-located molecular motor that mainly depolymerizes microtubules at the plus-end (34). Therefore, it is an attractive candidate to mediate delivery of MVB to the plasma membrane in neurons due to its specific localization and plus-end movement. Whether other kinesin(s) fulfill such a role in non-neuronal cells is still a matter of speculation. MVB transport to the plasma membrane likely requires the contribution of additional cytoskeletal systems, e.g. microfilaments. For example, Alix interacts with the ESCRT complex, but it also modulates subcortical actin dynamics (35). Also, clathrin localization to MVB may mediate their connection to subcortical actin (36). The establishment of strong bonds between cortical actin and MVB may result in their transport towards the plasma membrane following a “fishing net” model. In this view, actomyosin activation at the cortex would pull the actin-bound MVB towards the plasma membrane. The model implies that non-muscle myosin II pulls on the sides of the prospective docking site of the MVB on the inner leaflet of the plasma membrane. This occurs in a multicellular coordinated manner during dorsal closure in *Drosophila*. *Zipper* (NMII) pulls actin outwards along the cords of cells that define both edges of the amnioserosa, closing the gap among layers, which has been termed the “purse string” model (37, 38). A similar mechanism at subcellular scale is likely to participate in MVB recruitment.

MVB fusion with the plasma membrane is a process that requires Rab GTPases and likely membrane fusion/excision modules, e.g. SNAREs (for soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor). Several Rab proteins members, including Rab27a, Rab27b and Rab35 control this process in a cell-type-dependent manner. Rab7 and Rab11 seem to be involved in MVB fusion with lysosomes or autophagosomes (39), but its role in plasma membrane fusion is not clear. A recent study in which known Rab GTPases were systematically deleted did not show defects in Rab11-silenced cells (40). However, the same study identified Rab27a and Rab27b as part of the machinery that leads to fusion of MVB with the plasma membrane. Genetic deletion of Rab27a or its downstream effector Slp-4 (synaptotagmin-like 4) results in bigger MVB, whereas inhibition of Rab27b or its downstream effector exophilin-5 results in normally-sized MVB that do not translocate to the plasma membrane, remaining asymmetrically distributed in the central part of the cell (40).

Not much is known regarding the fusogenic machinery implicated in exosome release. It is likely that some member(s) of the SNARE family are implicated in this process. Early evidence of such involvement has been shown in K562 cells, in which VAMP7 and NSF

(which participates in SNARE disassembly) are involved in the fusion of MVB with the plasma membrane (41). However, in another cell line, MDCK, it has been shown that VAMP7 is required for the secretion of lysosomes but not for MVBs (42). In *Drosophila melanogaster* the R-SNARE, Ykt6, is required for exosome secretion (43). In the larval neuromuscular junction, the target-SNARE Syntaxin1A is involved in exosome release, although its v-SNARE partner has not been yet identified (44).

The lipid composition of the plasma membrane probably plays a role in MVB fusion, but whether plasma membrane lipid platforms trigger the targeting of MVB to the plasma membrane is unclear. It is well known that specific lipid components, e.g. cholesterol, sphingomyelin, phosphoinositides endow regions of the plasma membrane with preferential capability to bind SNAREs. In highly polarized cells, the phosphoinositides show a polarized distribution in the plasma membrane. They also influence the molecular organization of the plasma membrane by recruiting specific protein binding partners which are involved in the regulation of the cytoskeleton and in signal transduction cascades that control the tethering of vesicles (45, 46). PtdIns(4,5)P₂ play important roles in establishing and/or maintaining cellular polarity, such as in directional migration, in local events, such as in phagocytosis, or in polarized epithelial cells. PI(4,5)P₂ is a landmark for exocytosis. Vesicles synaptotagmins utilize PI(4,5)P₂ to orient to plasma membrane sites. Munc13 proteins undergo Ca²⁺ dependent recruitment to PI(4,5)P₂ domains (47). Syntaxin also binds PI(4,5)P₂ (45). Recruitment and regulation of PIP kinases and phosphatases by activated signalling receptors at the plasma membrane, can also provide a means to regulate the MVB trafficking. During virus budding, e.g. HIV, phosphoinositides and viral PI(4,5)P₂-binding proteins, e.g. Gag, mark the focal point on the plasma membrane for virus emergence and release (48).

Some SNARE molecules, e.g. SNAP-25, are heavily palmitoylated (49), and this directs them to lipid rafts, incorporating syntaxin-1 to these domains as well (50). A component of the ESCRT complex, Hrs, interacts with SNAP-25 (51), providing a possible mechanism of recruitment. Hence, ESCRT complex patches on the surface of MVB could interact with SNAP-25 to mediate fusion of the outer leaflet. However, such a mechanism remains speculative at this time.

4 Organizing polarized delivery of exosomes

Cell polarization facilitates asymmetric distribution of organelles and proteins during physiological processes such as cell division, migration, and morphogenesis. MVB delivery to the plasma membrane is guided by signals and cues that stem from the intrinsic polarization of the secreting cell and even participate in the polarization process.

4.1 Exosome secretion in immune synapses

Polarized cellular architecture is controlled by the movement of the microtubule-organizing center (MTOC), also called the centrosome, to one side of the cell. In T lymphocytes, the MTOC is reoriented toward the immune synapse (IS) that is formed at the interface between the lymphocyte and the antigen presenting cell (APC) (52). This event brings the Golgi apparatus, MVBs, and other vesicular compartments associated with the MTOC toward the

IS, thus favoring the directional secretion of soluble factors and exosomes to the APC (Fig. 1). Phosphoinositide accumulation coordinates F-actin architecture and defines the site for MTOC polarization, allowing the IS to fully mature (53). Diacylglycerol and PIP3 gradients shape cytoskeletal architecture at the IS (54), and it is conceivable that these gradients also control MVBs polarization. In this regard, downmodulation of DGK α protein inhibited the polarization of MVBs towards immune synapse and exosome secretion (55).

The reorganization of the vesicular compartment is an essential event to transport receptors and signaling molecules towards the synapse, and is also involved in their removal from the plasma membrane to downregulate the activation signal (52, 56). Regulated vesicle trafficking also participates in the effector function of the active T lymphocyte, controlling the polarized secretion of cytokines and lytic granules (57). During the formation of the IS, T cells translocate MVBs towards the APC. MVB polarization predates local secretion of miRNA-harboring exosomes into the synapse cleft, which are subsequently taken up by APCs (58). These microRNAs are functional in the target APC and likely condition their fate post-contact.

Using total internal reflection fluorescence microscopy (TIRFM), integrated with electron microscopy, and electron tomography, Choudhuri et al., observed how microvesicles loaded with TCR are transferred from the T cell to the APC during IS formation. That study also demonstrated that the ESCRT-I protein TSG101 was necessary for TCR sorting into microvesicles (59). Although the mechanisms that regulate the secretion of these microvesicles share proteins of exosome biogenesis, however these vesicles arise from direct budding of the plasma membrane, and therefore they could not be referred as exosomes.

Besides explaining the origin of the vesicles, these data point to the possible mandatory role of the IS in sustaining sufficient cellular confinement for the exchange of vesicles from the donor to the acceptor cell with minimal diffusion. Current evidence supports unidirectional transfer of exosomes from the T lymphocyte to the APC (58). T cell MVBs are dynamically polarized to the synapse, but MVBs from APC remain randomly distributed (58). This mimics the dynamics of the MTOC, which translocates to the contact area in the T cell but remains randomly positioned in the APC, supporting that the vesicle transfer occurs in one direction.

Comparison of the fate and the function of exosomes in recipient cells, when these exosomes are transferred through the IS or by direct addition to the recipient cell (58), supports the importance of polarized delivery of exosomes during IS. When acquired through the IS, exosomes seem to be fused with the cell surface, and exogenous miRNA activity is detected in recipient cells. In contrast, cells exposed to isolated exosomes without cellular contact present no exogenous microRNA activity, and the transferred exosomal protein can be washed by treatment with trypsin, suggesting that exosomes are adhered to the surface (58). It has been proposed that, depending on the origin of exosomes and the nature of the recipient cells, exosomes could be internalized by endocytosis, phagocytosis, or by direct fusion with the plasma membrane (60). Whatever the mechanism, the differences between exosomal transfer via synaptic and non-synaptic uptake support the notion that the

immune synapse promotes the directed secretion of exosomes by T cell and facilitates functional uptake of the miRNA into the APC.

Polarized secretion is not limited to exosomes; T cells can also secrete cytokines in a polarized manner. In fact, T cells release cytokines in a polarized and in a non-polarized fashion. IL-2 and IFN γ secretion are directed towards the IS, whereas IL-4 and TNF-alpha secretion occur in a non-polarized, multidirectional manner (61).

Viruses, e.g. HIV-1, have evolved to hijack cellular mechanism of cell-to-cell communication to promote their spreading. HIV-1 and other enveloped viruses use the exosome pathway and viral synapses for dissemination (62). The formation of the virological synapse promotes the reorientation of the MTOC and secretory organelles of the infected cell toward the cell junction and the consequent budding of HIV-1 at the sites of cell-cell contact (63). Besides HIV-1, other viruses, including non-enveloped viruses use the exosome pathway and ESCRT machinery to hide from the immune system (64–66).

4.2 Exosome secretion in neuronal synapses

Exosomes also participate in intercellular communication phenomena in the central nervous system (Fig. 1). They have been involved in the transmission of microRNA to neural cells (67) and also in microRNA released from depolarized cells (68). They also seem to participate in the transmission of pathogenic proteins (69–72). Mature neurons secrete exosomes and their secretion can be modulated by calcium and by glutamatergic synaptic activity (73, 74). In *Drosophila*, exosomes are vehicles of trans-synaptic transfer of proteins at neuromuscular junctions, e.g. Wnt. Secreted Wnt plays critical roles during synaptic development and plasticity, and it is transported across synapses by exosomes. Syntaxin 1A, Rab11, and its effector Myosin5 are required for proper exosomal release (44). How MVBs contribute to important biological functions, such as protein transport and signaling in neuronal cells has been recently reviewed (75). Many synaptic buttons contain MVBs in close contact with the presynaptic membrane (44, 76). As described above, synaptotagmin-4 is packaged in exosomes and controls retrograde signals that mediate activity-dependent synaptic growth (77), which mediates the mechanical and structural stabilization of the contact in an actin-dependent manner (78). Also, the bidirectional communication of exosomal miRNA permits the reciprocal control of the response of the presynaptic and the postsynaptic cell. For example, neuron exosomal miRNA controls the expression of the glutamate receptor GLT1 in glial cells (79). Such a mechanism likely constitutes an additional checkpoint for clearance of glutamate excess in the synaptic cleft. Oligodendrocytes in contact with neuronal axons secrete exosomes in response to glutamatergic stimulation, and these exosomes enhance neuron survival under stress (80). Neural activity triggers the transfer of oligodendroglial exosomes and their cargo to neurons. Thus, neurons themselves would regulate their supply of protective glia-derived exosomes.

4.3 Exosome secretion during cell migration

Migration is a polarized cellular process that opposes a protrusive front edge to a retracting trailing edge (Fig. 1). Actin-mediated forces sequentially promote cell protrusion, adhesion, contraction, and retraction (81). In general, MVB follow the distribution of the microtubules

in polarized cells. In migrating *Dictyostelium discoideum* cells, MVBs accumulate at the back of cells where they release their contents as exosomes and propagate chemotactic signals (32). Likewise, polarized T lymphocytes target exosome lipids and proteins to the posterior pole, the dwelling location of the Golgi and MTOC (82).

Besides neural development and function, Wnt signaling also depends on exosomes and MVBs to control cell polarity (83). Wnt signalling induces a Ca^{2+} -dependent release of exosomes containing pro-angiogenic factors, such as IL-6, VEGF and MMP2 in tumor cells favoring endothelial branching (84). Fibroblasts secrete exosomes that are internalized by breast cancer cells. Once they are internalized, they associate with Wnt11 and activate planar cell polarity (PCP) pathway signaling promoting cell polarity, that favors migration and metastasis (85) (43).

Invasive cancer cells extend invadopodia protrusions from their ventral surface into the extracellular matrix. Invadopodia are actin-rich membrane protrusions with a matrix degradation activity, and participate in degradation of the substrate at discrete sites (86). Invadopodia have recently emerged as sites of polarized secretion of exosomes (87), and their secretion is essential for invadopodia formation and function (Fig. 1). To promote the polarized delivery of exosomes containing matrix-degrading proteases, particularly membrane type 1 metalloprotease (MT1-MMP), it is critical that MVB dock at the invadopodia. The molecular mediators that control the docking of MVB to invadopodia sites are still unknown. Similar to synapses, invadopodia assembly also requires the polarization of the actin cytoskeleton, the microtubule network and the intracellular vesicle trafficking, suggesting that MVB docking mediators could be similar in both systems (87).

4.4 Exosome secretion by epithelial cells

Epithelial organ morphogenesis involves sequential acquisition of apical-basal polarity and development of a functional lumen (88). It has been described that the exosomes that are released from the apical surface are different from ones released from the basolateral surfaces (25)(Fig. 1). The asymmetry of secretion was also observed for the chaperone $\alpha\beta$ crystallin in retinal pigment epithelium. $\alpha\beta$ crystallin-carrying exosomes are secreted towards the apical, photoreceptor-containing side (89). The polarized delivery of exosomes has also been shown to be in transferring luminal peptidoglycan to lamina propria. Intestinal epithelial cells uptake peptidoglycan from the apical side and processed it in MVB for its posterior release via exosomes to the basal side of the cells (90). In *C. elegans*, apical secretion of Hedgehog-related proteins involves first their incorporation into the intraluminal vesicles of MVBs, and their subsequent release when MVBs fuse with the apical plasma membrane in a V-ATPase dependent manner (91). The existence of different populations of exosomes released from apical and basolateral cell surfaces not only demonstrates the existence of different subpopulation of vesicles, but also strongly suggests the existence of very specialized mechanisms to control the selective sorting of cargo into different vesicles.

5 Concluding remarks

Unlike shedding vesicles, exosomes are “stored” inside late endosomes until they are released to the extracellular environment. MVB maturation and translocation to the plasma

membrane depends on microtubules, actin and molecular motors that generate the forces required for these events. Finally, proteins involved in membrane fusion and fission control exosomal release and fusion with the target cell. Most of the studies on exosome secretion and function have been performed using exosomes isolated from cellular cultures or biological fluids. Comparatively less emphasis has been placed on the secretory cells and the process of MVB transport and docking at the plasma membrane. However, recent evidence indicates that, in some situations, exosome secretion occurs in a polarized manner. This is in fact important in several physiological contexts, e.g. processes that require intrinsic cell polarization such as cell migration and epithelial dynamics, and specialized cell to cell contacts. The polarized secretion of exosomes towards specialized cell-to-cell contacts such as cellular synapses may be functionally advantageous compared to multidirectional secretion. Such advantages include signal confinement or enhanced exosomal uptake by the receptor cells.

Future studies will undoubtedly unveil the molecular mechanisms that guide the spatial positioning of MVB. The role of phosphoinositides in membrane dynamics, including endocytosis, exocytosis, and vesicle trafficking, makes them suitable candidates to direct MVB positioning at specific focal points of the plasma membrane. Future studies employing super-resolution microscopy are needed to address spatial and temporal regulation of second lipid messengers in recruiting MVB to the plasma membrane for exosome secretion.

The polarized secretion of exosomes adds an additional layer of complexity to the function and physiological roles of exosomes. In the near future, a combination of genetics, high-end biochemistry and novel imaging techniques, such as high resolution tomography or single molecule imaging, will unveil the mechanical cues that are involved in MVB positioning to specific sites and the polarized secretion of exosomes.

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Figure 1. Polarized exosomal secretion in different cellular models

Immune synapse: The T cell positions its exosomes adjacent to the contact zone with the APC and drives polarized exosomal release. This is not incompatible with isotropic release of exosomes by T cell activation.

Neuronal synapse: The presynaptic portal releases synaptic vesicles (in purple) towards the post-synaptic button. The post-synaptic button releases exosomes to the cleft that may affect the pre- and post-synaptic zones to amplify or quench synaptic release and have additional effects in synapse stability and function.

Polarized migration: MVB are positioned at the rear and release exosomes that may contain chemotactic cues for other cells.

Invadopodia: MVB are juxtaposed to invadopodia and release exosomes that may contribute to matrix degradation and communication with neighbor cells.

Polarized epithelia may sort apical (light green) or basal (light blue) exosomes