To be or not to be... secreted as exosomes, a balance finely tuned by the mechanisms of biogenesis

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a balance finely regulated by the mechanisms of biogenesis.

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ABSTRACT

The release of extracellular vesicles such as exosomes provides an attractive intercellular communication pathway. Exosomes are 30-150 nm membrane vesicles that originate in endosomal compartment and act as intercellular mediators in both physiological and pathological context. Despite of the growing interest in exosome functions, the mechanisms responsible for their biogenesis and secretion are still not completely understood. This knowledge is yet capital as it controls the composition, and hence the function, and the secretion of exosomes. Exosomes are produced as intraluminal vesicles (ILVs) in very dynamic endosomal organelles that undergo various maturation processes in order to form multivesicular endosomes. The function of multivesicular endosomes will notably be balanced between exosome secretion and lysosomal degradation. In this review, we present and discuss each intracellular trafficking pathways that have been reported or proposed to regulate exosomes biogenesis with a particular focus on the importance of endosomal dynamics in the sorting of protein cargoes to exosomes and the secretion of multivesicular endosomes. A global picture reveals several key mechanisms that act mainly at crossroads of endosomal pathways acting as regulatory checkpoints of exosome biogenesis.

Summary Points:

. Biogenesis of exosomes is regulated by various checkpoints that scattered the endosomal pathway.

. A first checkpoint regulates the targeting of exosomal cargoes from the plasma membrane to the multivesicular endosome.

. A second checkpoint allows the sorting of cargoes to intraluminal vesicles of multivesicular endosomes via several sorting mechanisms that influence the fate of the compartment.

. A third checkpoint controls the balance between the secretion and the degradation of multivesicular endosomes.

. The intracellular pathways involved in exosome biogenesis are determinant for their function as they regulate the composition, the polarized release and the fate of exosomes.

ABBREVIATIONS LIST

Extracellular vesicles (EV), intraluminal vesicles (ILVs), multivesicular endosome (MVE), ADP-ribosylation factor 6 (Arf6), Endosomal Sorting Complex Required for Transport (ESCRT), Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1), post-translational modification (PTM), tetraspanin 6 (TSPAN6),

INTRODUCTION

All cells, from bacteria to human, release different types of extracellular vesicles (EV), that can be taken up by nearby or distant cells and modulate their activity. Hence, EVs can act as intercellular mediators in many physiological and pathological situations, including development, physiological communication (e.g. neurons, skin), immune response, cancer progression and metastasis, cardiovascular and neurodegenerative diseases(1). The origin, nature, size and content of these vesicles are diverse. Despite of the use of many different terminologies to identify EVs in the literature and a nomenclature that is still a matter of debate, two main classes of extracellular vesicles can be identified: microvesicles and exosomes(2). Microvesicles are generated by outward budding and fission of the plasma membrane and the subsequent release of these vesicles into the extracellular space(3). Their size ranges usually from 50 to 1000 nm in diameter, but can become larger (up to 10 µm) in the case of oncosomes. Exosomes originate as intraluminal vesicles (ILVs) by inward budding of the limiting membrane of maturing endosomes usually referred as multivesicular endosome (MVE) or multivesicular body (MVB). MVEs are able to fuse with the plasma membrane and release ILVs from 30 to 150 nm in diameter(4) in the extracellular environment as exosomes. While an increasing number of studies investigated the roles of EVs in cell-cell communication, less attention has been paid to the mechanisms involved in their biogenesis(5) and how they are regulated to modulate EVs secretion in producing cells.

In this review, we will only focus on exosome biogenesis and the generation and secretion of MVEs (further details on microvesicle generation can be found in more detailed review(3)). MVEs are part of the endosomal pathway that allows protein cargoes to be either recycled or degraded after their internalization(6). Therefore, MVEs cannot only be considered as the site of generation of the future exosomes but as a dynamic maturing compartment with various functions that results from the coordination of multiple intracellular molecular mechanisms. The endosomal pathway is scattered with several crossroads allowing either the recycling of protein cargoes to the plasma membrane or the Golgi apparatus or their sorting to ILVs. MVEs form then a crossroad that will target cargoes sorted on ILVs either to secretion or to lysosomal degradation. Each sorting step in the endosomal pathway can be viewed then as a regulatory checkpoint. As a consequence, that the different mechanisms involved in exosome biogenesis, from the intracellular trafficking of cargoes to MVE including their sorting into ILVs to the targeting of MVEs toward the plasma membrane instead of toward lysosomes act as regulatory elements of exosomes biogenesis. We will discuss how each step of the endosomal pathway can influence exosome biogenesis with a particular focus on the balance between endosomal degradation and secretion.

EXPRESSION OF CARGOE AS FIRST REGULATORS OF EV BIOGENESIS

Cargoes destined for exosomal secretion can be viewed as the first regulators of exosome formation as the presence of a particular cargo at MVEs can induce the recruitment of specific machineries that will lead to ILVs formation. This idea is supported by the observation that ectopic expression of MHC Class II in HeLa cells(7) or its forced expression in intestinal epithelial cells after stimulation by γIFN or expression of its transactivator CIITA increased the release of extracellular vesicles (8). This

increased secretion can be attributed to the increased generation of MVEs and the recruitment of sorting machineries by the cargoe that will promote ILV (or future exosomes) generation. Expression of another exosomal cargoe, PMEL, a specific melanosomal protein, in Hela cells induces indeed its sorting to ILVs by recruitment of specific sorting mechanisms(9) and generation of subpopulations of ILVs (10) and the production of exosomes (G. van Niel unpublished data) (10)(11). Similarly, overexpression and depletion of syndecan from MCF-7 cells respectively increase and decrease the recovery of exosomal markers (12). In this context, the regulation of the expression of a given cargo, which is either cell type specific or induced by external stimuli, is the first regulatory elements that will modulate exosome biogenesis. It should be noted that a default of exosome secretion could then reflect an impaired expression of exosomal cargoe. It could also result from an inhibition of MVE secretion or a defect of ILV generation. To avoid such misinterpretation, cargo expression, exosome recovery and exosome generation as ILVs in MVE should be investigated in parallel. As well, isolation and investigation methods used for the study of EVs can lead to the selection or the enrichment of a given subpopulation of exosome (13). Therefore, a particular attention should be given to the methods and the cellular models employed in each study to clearly conclude on a defect of ILV generation or of MVE secretion.

TARGETING CARGOES TO MULTIVESICULAR ENDOSOMES

To be secreted on exosomes, cargoes need to reach the endosomal pathway before being sorted to intraluminal vesicles. Muted version of PLP retained in the Endoplasmic reticulum, for example, are unable to reach the endosomal pathway and are not secreted on exosomes (14). Exosomal membrane cargoes can reach endosomes by being internalized from the plasma membrane or by direct targeting from the Golgi apparatus.

Known cargoes of exosomes such as PMEL (9) or APP (15) are first transported to the plasma membrane from where they are internalized to reach endosomes. Endocytosis of proteins can involve different pathways. The most studied is clathrin-dependent endocytosis, which requires the polymerization of a clathrin coat on the cytoplasmic face of curved plasma membrane and the subsequent invagination and scission of the vesicles, with this last step mediated by the GTPase dynamin (16). This pathway is notably employed by mature MHC II to reach $Arf6^{+}Rab35^{+}EHD1^{+}$ tubular endosomes (17) and by EGFR (18). Of note, such endocytosis is dependent on ubiquitination of the cargoes (19),(20) that targets them to degradation and not exosomal secretion. Other pathways, defined as “clathrin- independent endocytosis” (CI), include caveolae vesicular mediated endocytosis (also dynamin dependent) and the clathrin- and dynamin-independent carriers (CLICs) tubular intermediates (21). Such pathways allow internalization of different cargoes such as CD44 and GPI-anchored protein (22)(23) or Galectin 3 (24) that are all found on exosomes. The relevance of CI pathway in the exosomal targeting of these cargoes is not proven yet although small GTPases such as the ADP-ribosylation factor 6 (Arf6) and Rho subfamilies members RhoA and Cdc42, which have been implicated in regulating different pathways of CI endocytosis, are all found in extracellular vesicles and Arf6 and Cdc42 were found to modulate exosomes and microvesicles secretion (25), (26), (27). Finally, flotillins, which are commonly used as exosome markers, can regulate the

endocytosis of specific cargoes, but the underlying mechanisms are still poorly understood. Flotillins may favor the clustering of the cargoes, therefore promoting their endocytosis that in some cases may require the presence of clathrin (28). All these internalization pathways will deliver their cargoes to early endosomes (EE) (6) providing a first checkpoint to target cargoes to MVEs and exosomes.

Early endosomes appear as vacuolar compartments characterized by the presence of the small GTPase RAB5 from which thin tubular structures are pulled to generate recycling endosomes (RE). Cargoes targeted to recycling endosomes will be targeted back to the PM while cargoes destined to MVEs will accumulate on vacuolar subdomains of the EE and will be sorted into ILVs. Hence, cargoes that are destined for recycling to the plasma membrane will likely not be enriched in exosomes, unless their recycling is impaired as in case of transferrin receptor in reticulocytes (29). Such balance between recycling and targeting to MVE can be seen as a second checkpoint that will regulate exosome biogenesis. Among the proteins that regulate this crossroad between recycling and intraluminal sorting, the PDZ protein syntenin is an ideal suspect. In endosomes, syntenin interacts with syndecans and support their recycling to the plasma membrane trough a mechanism that requires the production of $PI(4,5)P_2$ and the small GTPase Arf6 (30). Alternatively, syntenin, together with the ESCRT accessory protein ALIX, can dispatch syndecans to ILVs (12). Syndecans, syntenin and ALIX assemble in a tripartite complex that is able to drive not only their own exosomal release, but also the release of CD63 and HSP70. Interestingly, Arf6 and his effector PLD2 also control budding of ILVs and biogenesis of exosomes via the syndecan-syntenin-ALIX pathway (27). Although it remains to be determined if recycling of syndecans affects directly syntenin-exosomes biogenesis, the fact that the same proteins play a role in both recycling and intraluminal sorting clearly illustrates the role of EE as the first “sorting station” of the cell, in which the fate of a cargo is primarily decided, and how this process needs to be tightly regulated. One could propose then that the “internalization” checkpoint and the “recycling” checkpoint, by regulating the localization of cargoes between the respective site of generation of exosomes and microvesicles, i.e. MVE or plasma membrane, may modulate a potential balance between exosome and microvesicle biogenesis. In this context, Arf6, by being involved in both exosome (27) and microvesicle (26) biogenesis, may control such balance between the generation of both subpopulations of EVs.

Direct transport of proteins from Golgi to endo-lysosomal system has been mostly studied for the transport of lysosomal proteins. Transmembrane proteins possess a small amino acid motif (i.e., tyrosine- or dileucine-based motifs) that will be recognized by clathrin adaptor proteins allowing the formation of Golgi derived clathrin coated carriers that will then fuse with the endosomes (31). The relevance of a direct transport from Golgi to MVEs and ILVs is illustrated by the protein GPRC5, a member of GPCR family proteins, which uses GGA1 carrier vesicles to be sorted on exosomes (32). Soluble proteins need to bind specific receptors (e.g. mannose phosphate receptor, sortilin) to be transported from Golgi apparatus to MVE. Once in endosome, the cargoes protein is released while the receptors usually recycle back to the Trans Golgi network by retrograde transport (31). Retrograde transport can then be seen as an alternative route from endosome that cargoes should avoid to end up in ILVs. This additional crossroad represents a third checkpoint that could be regulated by parkin, an E3 ubiquitin ligase that is found mutated in Parkinson’s disease (PD). Parkin would regulate such

balance between retrograde transport and targeting to ILVs by acting both on retrograde transport (through the retromer complex) and on inward budding and exosome secretion (33). Interestingly, sortilin, a receptor involved in anterograde transport from Golgi and a known cargoe of the retrograde transport (34) has also been shown to be secreted on exosomes and to modulate exosome biogenesis (35), but the trafficking mechanisms underlying such regulation needs to be further investigated.

ILV BIOGENESIS in MVEs

Exosome biogenesis occurs in maturing endosomal compartment. Importantly, maturation of early endosomes into late endosomes requires switching of the RabGTPase RAB5 with RAB7 (6), which can be blocked by overexpression of a constitutively active mutant form of RAB5 (Q79L). This mutant induces fusion of early endosomes that are still able to form ILVs despite they appear enlarged (14). For this reason, RAB5 mutant expressing cells have been often used to visualize ILVs and to study the sorting of cargoes to ILVs (12) (14) (36) (37). In addition depletion of RAB5 in Hela (7) cells or RAB7 in MCF cells (12) (but not in Hela (7)) have been shown to modulate exosome secretion, although they may not have a direct role in exosome biogenesis but rather a global effect of endosome homeostasis. Of note, the precise step of maturation of MVE at which they would be preferentially secreted is still unknown.

Within maturing endosome, likely concomitantly to Rab5/Rab7 switch, future exosomes are generated as ILVs through a process that requires the invagination and fission of the endosomal membrane to form an intraluminal vesicle. The first mechanism that was described for this process is the Endosomal Sorting Complex Required for Transport (ESCRT). ESCRT complex is composed by four different subcomplexes, ESCRT-0, -I, -II, -III and associated proteins such as ALIX, VTA1 and ATPase VPS4 (38). These subunits act together in a stepwise process that starts by the recruitment of ESCRT-0 at endosomal limiting membrane through ubiquitin moieties attached to the cytoplasmic domain of the transmembrane cargoes to sort. Importantly the cargoe (and Pi3P) will recruit the sorting machinery, strengthening the notion that the cargo is the first regulator of ILV biogenesis. ESCRT-0 and ESCRT-I will then cluster the cargoes under flat clathrin coat creating a subdomain of the endosomal membrane that will bud into ILV. The flat clathrin coat is thought to avoid cargoes diffusion and possibly their targeting to alternative pathways such as recycling or retrograde transport (39). This coat must be removed, potentially by the exosomes-enriched protein V-ATPase HSC70 (40) (41), to allow the recruitment of ESCRT-II and –III, the latter being responsible of membrane scission together with VPS4. During this process, ESCRT-III recruits deubiquitinating enzymes that remove ubiquitin tag from cargo proteins prior to the release of newly forming ILVs in the lumen of MVE. However, ubiquitinated proteins can be still found in exosomes suggesting that deubiquitination is not a critical step in exosome biogenesis (42). Of note, the accessory protein ALIX, that intereact with the escrt-I subunit TSG101, provides an alternative route by recruiting directly ESCRT-III and VPS4 (12).

The role of ESCRT machinery has been deeply investigated by Colombo et al. through RNA interference screen targeting of 23 ESCRT and ESCRT associated proteins in Hela cells and

identifying 7 proteins that affect exosome secretion (43). While depletion of ESCRT-0 and I STAM, HRS and TSG101 reduced exosome secretion, depletion of ESCRT-III CHMP4C, VPS4, and accessory molecules VTA1 or ALIX increased exosome secretion. It should be noticed that, in the same study, depletion of ALIX increased MHCII secretion on exosomes but had variable effects on CD63 and HSC70 levels, suggesting that ALIX may affect more the composition of exosomes rather than global exosomal secretion. As the study was mainly focused on the analysis of exosomes once they were released from HeLa cells expressing the CIITA transactivator, it is still unclear which steps of exosome biogenesis were affected and whether it is specific to the forced expression of MHC II. But ESCRT-0 protein HRS has also been involved in exosome secretion in dendritic cells (44), in the release of exosomal Wnt3 (36) and its depletion was shown to globally decrease exosomes number as measured by Nano particles tracking Analysis (45). More recently, inhibition of VPS4 in HEK293 cells has been shown to decrease release of different subpopulation of EVs, corresponding to CD63 enriched exosomes and CD9 enriched microvesicles (46). Interestingly, electron microscopy has shown that Hela cells depleted for HRS display MVBs containing small ILVs (below 40nm of diameter) (10). It cannot be excluded then that, due to their small size, some subpopulations of exosomes are below the threshold of detection of some methods of investigation or still lack specific marker to be detected on western-blot.

The ESCRT machinery can be partially (via ALIX) or completely dispensable for the generation of ILVs. ESCRT-independent biogenesis of ILVs was firstly revealed using simultaneous depletion of components of the four ESCRT subcomplexes (47). Interestingly, electron microscopy has shown that ESCRT independent ILVs are enriched in the tetraspanin CD63 (10) (47). Tetraspanins are a family of proteins with four transmembrane domains. Tetraspanins share a similar structure and organize as highly dynamic membrane microdomains called tetraspanin enriched microdomains (TEM), where tetraspanins interact with each other and with other transmembrane or cytosolic signaling proteins (48). Different tetraspanins have been proposed to have a role in ILVs formation. Bone marrow dendritic cells (BMDCs) from CD9 knockout mice secrete less exosomes compared to wild-type mice (49), while expression of CD9 and CD82 promote the release of β-catenin in exosomes (49). In a mouse model of breast cancer, the release of Wnt11 on exosomes from cancer-associated fibroblast is dependent on tetraspanin CD81 (50). Expression of another tetraspanin, Tspan8 has been shown to change exosomal protein and mRNA content in rat adenocarcinoma cells (51). Finally, the tetraspanin CD63, which is particularly enriched in exosome membranes, plays an important role in exosome biogenesis in multiple cell types. CD63 is required for the generation of small ILVs in Hela cells depleted for HRS (10) and has been recently involved in the biogenesis of exosomes in fibroblasts from Down syndrome patients (52). CD63 associates and targets Epstein-Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) to ILVs and is critical for its secretion on exosomes (53) (54). In melanocytes, CD63 is required for the sorting of the melanosomal protein PMEL to ILVs (55), a process that involved the targeting of Apolipoprotein E to ILVs and exosomes but not the ESCRT machinery (56).

In addition to proteins, lipids have also been involved in exosome biogenesis. The first ESCRT-independent mechanism reported for exosome biogenesis was identified in Oli-neu cells and requires

generation of the sphingolipid ceramide through hydrolysis of sphingomyelin by neutral type II sphingomyelinase (14). Ceramide could create specific lipid microdomains and induce negative membrane curvature that will give rise to ILVs and exosomes enriched in ceramide. In addition a metabolite of ceramide, the sphingosine 1-phosphate, has recently been shown to continuously activate G_i-protein coupled sphingosine 1-phosphate receptor and to regulate in this way the sorting of cargoes into exosomal ILVs (57). Exosomal membranes can also contain lipid rafts, specific lipid microdomains enriched in cholesterol and glycosphingolipids, which can contribute to ILV formation and sorting of raft-associated molecules such as Glycosyl PhosphatidyInositol (GPI)-anchored proteins (58). In a similar way, the Ca^{2+} -dependent phospholipid-binding protein Annexin A2 binds to lipid raft and is transported along the endocytic pathway where it is sorted into ILVs (59). Diacylglycerol (DAG), another lipid second messenger, also seems to have a role in exosome formation. In fact inhibition of DGK, an enzyme that metabolizes DAG into phosphatidic acid has been shown to induce release of exosomes in T-cells (60). Finally, bis(monoacylglyceryl)phosphate (*BMP*), formerly termed lysobisphosphatidic acid, (LBPA), is specifically enriched in late endosomes such as MVEs and can recruit ALIX to generate ILVs in vitro and in vivo (61). Phospholipase D (PLD), which hydrolyzes phosphatidylcholine to generate choline and phosphatidic acid, has also been involved in the generation of exosomes via the syntenin-ALIX pathway (27).

Such proteolipid interactions, which are well known to induce membrane curvature (62), illustrate the interconnections between the multiple sorting machineries acting at MVEs. In this context, a recent structural study of the tetraspanin CD81 revealed a cone-like structure with an intramembrane pocket that can bind cholesterol (63). As proposed for the cone-shaped ceramide (14), clustering of several cone-shaped tetraspanins could then induce inward budding of these microdomaines. Another interrelationship between sorting mechanisms is illustrated by the known interaction of CD63 with syntenin (64), the regulation of CD63 by the Alix-syntenin pathway (12) and the formation of highly dynamic microdomain between different tetraspanins such as CD63, CD9 and CD81 (65) (13). Moreover, the syntenin-ALIX pathway in MCF (12) cells and the sorting of CD63 in HeLa cells (66) are both regulated by ceramide. But interconnections between the reported sorting machineries seems to be highly dependent on the cell type under investigation as the sorting of PMEL through ApoE, a soluble apolipoprotein with particular affinity for lipids, depends on CD63 but not on ESCRT or ceramide (56) in pigment cells.

Similar intersection or coordination also exists between ESCRT-dependent and –independent mechanisms. Non ubiquitinated cargoes can be trapped in microdomains formed by the clustering of highly ubiquitinated tetraspanin Cos. This ubiquitination “in trans” will allow the recruitment of the ESCRT machinery and “canonical” formation of ILVs in which non-ubiquitinated cargoes are also sorted (67). ESCRT-dependent and ESCRT-independent mechanisms for ILVs biogenesis are tightly coordinated at a single MVE in Pigment cells by the tetraspanin CD63 (55). ESCRT-dependent and ESCRT-independent mechanisms will give rise to a heterogeneous population of ILVs in term of size but also in term of cargoes (10). The variety of sorting mechanisms in a single cell type likely explains the heterogeneity in terms of size and composition of produced exosomes.

Globally both ESCRT-dependent (at least ESCRT 0 and I that are also involved in syntenin-Alix pathway) and ESCRT–independent mechanism can participate at different extent in exosome biogenesis and provide a central checkpoint in this pathway. ESCRT-independent and semi-dependent mechanisms seem nevertheless favoring ILVs release as exosomes while the conventional ESCRT dependent mechanism seems more dedicated to degradation.

MODIFICATION OF CARGOES FOR THEIR SORTING TO EXOSOMES

As main recruiters of sorting machineries at MVEs, any modification of cargoes fated to exosomal release may be determinant for their sorting and their release on exosomes. A major regulation of the sorting of proteins to exosomes is their post-translational modification (PTM). Interestingly, different PTM can compete to determine the fate of the same cargo (68). A major PTM is ubiquitination that consists in the binding of ubiquitin moieties to cysteine present in the cytoplasmic domain of transmembrane protein or present and accessible on soluble proteins. Its role in the sorting of cargoes into ILVs destined for lysosomal degradation has been extensively studied and involved the recognition of the ubiquitinated cargoes (e.g. EGFR, MHCII) by ESCRT machinery. On the same line, ubiquitinated MHCII is targeted to MVEs fated for lysosomal degradation and sorted to ILVs likely through an ESCRT-dependent mechanism. Contrarily non ubiquitinated MHCII can be secreted on exosomes and its sorting to ILVs involves incorporation into CD9 containing membranes likely in an ESCRT-independent manner (69). On the same line, exosomal secretion of SIMPLE (70) is enhanced after mutation of its binding site for ubiquitin ligase, suggesting that ubiquitination negatively regulates SIMPLE secretion on exosomes. This set of studies strengthens the notion that ESCRT-dependent and ESCRT-independent mechanisms are respectively associated with exosomes release and lysosomal degradation.

Other PTM that can be important for the incorporation of cargoes in exosomes are addition of a small ubiquitin-related modifier (SUMO), as reported for heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1), a key regulator for the recognition of specific miRNAs and their loading into exosomes (71); phosphorylation, as in the case of Annexin A2 (59), and oxidation, as in the case of γ-synuclein (72). Moreover the detections of many glycosylated proteins in exosomes suggest that glycosylation may also have a role in exosomal sorting, despite this needs to be further investigated (68). Finally ISGylation, a PTM consisting in the addition of the small ubiquitin-like protein ISG15, has been recently shown to negatively regulate exosome release (73), although it would rather act on the sorting machinery and the fate of the MVE than on the cargoe itself.

In addition to PTM, protein cargoes can go through proteolytic cleavage before being incorporated into exosomes. Heparanase, the only mammalian enzyme able to cleave heparan sulfate internally, was identified as a stimulator of the syndecan-syntenin-ALIX pathway (74). Indeed, endosomal heparanase trims the heparan sulfate side chains of syndecans. Such trimming would favor clustering of syndecan and its proteolytic processing to generate “SDC-CTF” that is sorted to ILVs. Such processing may reinforce syndecans-syntenin interaction therefore favouring ILV budding. Similar coordination between processing and sorting is well illustrated in melanocytes with the protein PMEL (75). PMEL is cleaved by the secretase BACE2 at the limiting membrane of pigment cell specific MVE. The two

cleavage products of PMEL, its luminal domain and PMEL-CTF are taken in charge by distinct sorting machinery within the same compartment. While PMEL-CTF is sequestered in an ESCRT-dependent manner into the clathrin coat at the limiting membrane of the compartment and destined for lysosomal degradation, the luminal amyloidogenic domain of PMEL is loaded on the surface of ILVs (55) and can be secreted on exosomes (56) in a CD63 –dependent manner. Despite the role of CD63 in the loading of luminal domain of PMEL into ILVs is still not completely clear, depletion of CD63 induces the targeting of full length PMEL to the ESCRT dependent pathway, suggesting that this tetraspanin is a key coordinator of the interrelated processing and sorting of cargoes fated for exosomal release.

One can expect that the recruitment and the contribution of these different sorting pathways in exosomes biogenesis are not only dependent on the PTM or processing of the cargo to be sorted but will also depend on the cell type, the cellular homeostasis and the pathological conditions. But any enzymes involved in the modification or the processing of the exosomal cargoes can be considered as a regulator of exosomes biogenesis.

BALANCE BETWEEN DEGRADATION AND SECRETION OF MVEs

The main fate of a MVE is to fuse with lysosomes to degrade its content. Therefore MVEs must avoid lysosomal degradation in order to release their ILVs as exosomes. Several studies in which inhibition or blocking of lysosomal function induces exosome secretion support the hypothesis of the existence of a balance between lysosomal degradation and exosome secretion. For instance, treatment with Bafilomycin A1, a lysosome inhibitor, increases EV secretion (73) 74) or exosomal secretion of specific cargoes such as α-synuclein (77). On the same line, inhibition of V-ATPase in *Caenorhabditis elegans* has been shown to trigger apical secretion of Hedgehog protein (78). Such balance seems to be highly relevant in pathological conditions where lysosome activity is affected such as lysosomal storage diseases or neurodegenerative diseases. For instance, Niemann-Pick Type C (NPC) disease is caused by mutation in the cholesterol transporter NPC1 and is characterized by accumulation of cholesterol in endosomes and lysosomes. One study showed that NPC1 mutations or inhibition with drugs (U18666A) enhance secretion of cholesterol in exosomes (79), confirming, as shown in the first studies on exosomes (80), that exosomes is a way to eliminate compounds, such as cholesterol, that accumulate in the cell. Neurodegenerative diseases are often characterized by an impairment of lysosome function and an intracellular accumulation of toxic protein has been proposed to provoke their secretion extracellularly (81). For instance, accumulations of APP fragments Aβ and Tau are typical features of Alzheimer disease (AD) and both proteins were found in EVs released from AD cell culture models, in EVs isolated from CSF, brain extracellular space, and blood samples from AD patients (82,83). There is, so far, no direct evidence of a causal connection between intracellular accumulation and the release of these pathological proteins in EVs, but such pathway would certainly compensate for their impaired degradation inside the cells and therefore contribute to the maintenance of cellular homeostasis.

Mechanisms that prevent lysosomal degradation in favor of exosome secretion provide then a powerful checkpoint to regulate exosomes biogenesis but they remain yet poorly understood (81). As suggested before, endosomal sorting machineries seems to drive MVEs either to secretion or to

lysosomal degradation. New regulators of sorting machineries at MVEs reinforce their regulatory role in the balance between secretion and degradation. ISGylation of TSG101 (73) inhibits exosome secretion while mutations that impair ISGylation enhance exosome secretion. Such regulation is counterbalanced by the fusion of MVE with lysosomes establishing a first mechanism that can decide the fate of an MVE between fusion with lysosomes and fusion with PM. It also supports the notion that the first subunits of the ESCRT machinery can be employed in secretory and degradative MVEs, it would be then interesting to know whether ISGylation of TSG101 is determinant to recruit either conventional ESCRT machinery for degradation or ALIX for secretion. The tetraspanin 6 (TSPAN6) has also being recently involved in the regulation of this balance between lysosomal degradation and exosomal secretion. Overexpression of TSPAN6 slows down lysosomal degradation of APP-CTF and enhances its secretion on exosomes likely by recruiting syntenin (84). It remains also to determine if, in a cell type dependent manner, there is a clear separation of ESCRT-dependent and ESCRT-independent sorting associated with distinct subsets of endosomes fated respectively to degradation and secretion.

How then a given sorting can act on the motility and the targeting of a subpopulation of MVE? If both pathway can act concomitantly on the same MVE, one can propose that the aboundance of a given cargo or its PTM will favor the recruitment of one sorting mechanism rather than other and dictate the fate of MVE. Finally, as observed in specialized cells such as melanocytes, ESCRT-independent and ESCRT dependent could act sequentially, ESCRT independent acting first while cargoes fated for ESCRT dependent sorting are sequestered into clathrin coat at the limiting membrane. Only early MVE would be then able to be targeted for secretion. The cell type and its maturation state would be of importance as shown for dendritic cells that use different sorting mechanisms according to their maturation stage and reticulocytes that use different mechanisms for exosome secretion during their maturation into erythrocytes. Finally the only feature that has allowed to discriminate subpopulations of MVEs with apparent distinct fate is their content in cholesterol (85).

In addition to lysosomes, MVEs can fuse with autophagosomes forming an amphisome (86). This organelle will then fuse with lysosome in order to allow the degradation of their content, in a process called macroautophagy. Therefore, a similar balance between exosome secretion and autophagy may exist and would be dependent on external stimuli such as starvation. Supporting this hypothesis, it has been shown that the prion protein (PrP) can promote exosome secretion by inhibiting autophagosome formation through interaction with caveolin 1, a suppressor of autophagosome formation (87). Moreover, inhibition of the kinase PIKfyve, that is also involved in autophagy, increase secretion of a subpopulation of “autophagic” exosomes enriched in autophagic proteins but not in “classical exosomal markers”, again suggesting that impairment of autophagy can be balanced by secretion (88). Interestingly different autophagy proteins have recently been shown to regulate exosome secretion, such as ATG12-ATG3 complex that control both autophagy and exosome secretion through interaction with ALIX (89), or ATG5 which induces sequestration of a subunit of V_1V_0-ATPase in ILV inhibiting ATPase activity and therefore promoting MVEs acidification and exosome secretion (37). Additionally, the tetraspanin CD63 has recently been proposed to coordinate both autophagic and endosomal processes and to regulate exosomal secretion of Epstein-Barr virus-encoded LMP1 (90).

Finally, it is noteworthy that not only MVEs but also lysosomes and autolysosomes can secrete their content in the extracellular environment, included remaining ILVs. Despite these pathways could use different machineries, they cannot be entirely separated but they can all contribute to maintain cell homeostasis.

TARGETING MVE TO THE PLASMA MEMBRANE FOR SECRETION

Regardless of their fate, MVEs have to be transported before fusing with lysosomes or plasma membrane. In general intracellular transport of organelles requires association to the cytoskeleton together with the action of molecular motors and small GTPases, and their fusion involves SNARE proteins (91,92). In this emerging topic, the precise role of these machineries involved in exosome secretion would benefit from further investigations to be clearly understood.

Several RAB proteins have been involved in exosome secretion. For instance, use of a dominant negative mutant of RAB11, a GTPase mostly known for its role in recycling of cargoes from EE to PM, decreases secretion of TfR and HSC70 in exosomes from leukemia cell line K562 (93); while in Drosophila S2 cells, RAB11 depletion inhibits secretion of EVs bearing wingless (94) or Evi (95). In addition, depletion of either RAB11 or RAB35 in retinal epithelial cells, decreases exosomal secretion of flotillin and anthrax toxin (96). Other studies confirmed the involvement of RAB35 in exosome secretion from primary oligodendrocytes (97) or oligodendroglial cell line, where RAB35 seems to have a role in the docking and tethering of MVEs to the plasma membrane (98) . An shRNA based screen in HeLa cells expressing MHC class II molecules showed that silencing of RAB2B, RAB5A, RAB9A, RAB27A and RAB27B decreased CD63, CD81, and MHC class II secretion on exosomes (7). More in detail, the same study shows the involvement of RAB27A/B in the docking of MVBs to the PM and several following studies have confirmed in different cell lines the reduction in exosome secretion after RAB27A silencing and this strategy is now commonly used as a way to modulate exosome secretion(45,99–101). Finally, RAB7 has been involved in secretion of syntenin-ALIX exosomes by MCF7 tumors (12), whereas its depletion does not affect exosome secretion in HeLa cells (7). The fact that both early and late endosomal Rab proteins have a role in exosome secretion suggests that, according to the cell type, different subpopulations of MVEs originating from either early or recycling endosomes or from late endosomes are employed to release ILVs as exosomes. Alternatively, the involvement of so many RABs protein acting on the various compartments that scatter the endosomal pathway would suggest the interdependency of the different steps controlling the endosomal pathway in the regulation of exosomes biogenesis.

The importance of the cytoskeleton is supported by the specific location of the release of exosomes at the plasma membrane as in the case of immunological synapses in antigen presenting cells (69,102) or invadopodia (45) in cancer cells. The microtubules network would be of prime importance to transport MVEs to the site of secretion in coordination with molecular motor. RAB7 is a key regulator of MVEs motility as it can interact with dynein motor through its effector RILP favoring the transport of late endosomes toward the microtubules organizing center (MTOC) (103) where lysosomes localize. Such interaction is regulated by the ubiquitination state of RAB7 (33) and the presence of cholesterol in the limiting membrane of MVEs (104). This suggests that the composition of the limiting membranes

of MVEs may modulate the fate of these MVEs by acting on the motility of MVEs. It would also shed new light on the relationship between the fate of MVEs and the associated endosomal sorting machineries that modify the composition of the limiting membrane of MVEs by generating ILVs. Actin cytoskeleton would also affect exosome release as it is required for the docking and the fusion of MVEs with the plasma membrane. Actin regulatory protein cortactin has been shown to promote exosome secretion by regulating both trafficking and docking of MVEs to the plasma membrane together with the small RAB GTPase RAB27A and coronin1b (105). At this step, the machineries involved in docking and fusion of MVEs with PM are still not completely known, although some RAB GTPases, SNARE proteins and synaptogamin family members participating in this process have been identified. As previously mentioned, RAB27A and RAB27B (together with their respective effectors, synaptotagmin-like protein 4 and exophilin 5) act in the docking of MVEs to PM in order to promote their fusion (7) and this mechanism requires the rearrangement of actin cytoskeleton (105). The SNARE protein VAMP7 has been involved in exosome secretion in leukemia cell line K562 (106) and expression of a form of VAMP7 that inhibits SNARE complex formation decrease exosome secretion. Another SNARE, YKT6 has been shown to be required for exosome release in HEK239 cells (36) and in A549 lung cancer cells (107). In C. elegans the Ras-related GTPase homolog Ral-1 is involved in MVEs biogenesis and in their fusion with PM together with the SNARE syntaxin 5 (108). Recently phosphorylated SNAP23 has been shown to enable exosome release (66,109). Of note, new tools to directly vizualise exosomes secretion in live cells, such as CD63-pHluorin, open new avenues to map and understand the release of exosomes at the scale of a single cell. In this context, the release Ca^{2+} in the cytoplasm seems differently required for the activation of SNARE complexzq involved in MVE-PM fusion, revealing cell type specific contribution of this know regulator of exosome secretion (66,110–112). This last checkpoint is determinant in exosomes function, as it will orientate exosomes secretion toward the right cellular partner of a synapse (69) or toward the right body fluids or biological barrier to reach its final destination.

CONCLUSION

The multiple checkpoints that have been described here as potential regulator of the biogenesis of exosomes are all subjected to regulatory pathways that can be stimulated or inhibited under physiological or pathological conditions (113). This implies that exosomes biogenesis and secretion are likely more part of an inducible pathway that is modulated according to the stimuli received by the producing cells than a constitutive pathway. Once released in the extracellular space exosomes can reach body fluids such as blood that travel through the whole body supporting their targeting to destinations very far from their site of production. Alternatively, exosomes can stay closely associated to the cells that produced them through a mechanism that requires presence of tetherin (76). The machineries involved in the biogenesis of exosomes, by modulating their composition, will be then critical to specify their uptake by receiving cell types or to regulate their destination, as shown in vivo for tumor exosomes that can reach and stop in different organs according to the set of integrins they contain (114). Once at destination, the machineries that have been involved in their biogenesis in

producing cells will be also determinant in recipient cells to modulate exosome fate and to induce signaling cascade or to provide a trophic support.

the authros declare no conflict of interest

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Figure Legends.

Figure 1: Intracellular trafficking checkpoints involved in exosome biogenesis.

Letters in white squares indicate the different checkpoints. Blue arrows highlight the trafficking steps favoring exosome bioegenesis and the red arrows the trafficking steps impairing exosome biogenesis. Green arrows represents alternative release mechanisms. Exosomal cargoes reach endosomal compartment through endocytosis or by direct transport from Golgi apparatus (a and a'). Before being sorted into ILVs the cargo need to avoid recycling to PM or Golgi (b and b'). Once formed (c and Figure 2), MVEs have to escape different fates such as fusion with lysosomes or autophagosomes (d) and be transported toward the plasma membrane in order to fuse with it and secrete exosomes in the extracellular environment (e). Main reported regulators of each step are indicated at each regulatory step.

Figure 2: Sorting mechanisms at MVE involved in exosome biogenesis.

Bold arrows outside of the MVE indicate the main sorting mechanisms reported so far. Dashed arrows within the lumen of MVE propose potential intersection or coodination between sorting mechanisms. Four main sorting mechanisms has been reported so far for transmembrane proteins, the classical

ESCRT machinery (a), the tetraspanin microdomains (b), the syntenin/Alix pathway (c) and the Ceramide pathway. Posttranslational modifications are key regulators allowing controlled targeting of a modified cargoe (ubiquitination, cleavage) to a specific sorting mechanisms (e and e').

Figure 3: The balance between degradation and secretion of MVEs.

A key checkpoint in the secretion of exosomes is the balance between lysosomal degradation of MVE and their targeting to the plasma membrane for secretion. Reported regulators influencing each scale of the balance are indicated. Of note it remains to be determined if targeting toward degradation or secretion is regulated by the generation of distinct subpopulations of MVEs by abundance of a given cargoe or recruitment of distincts sorting mechanisms, by the maturation of the MVE or by posttraductional modification of MVE associated proteins. Such balance is notably important in lysosome storage disorder and amyloid associated neurodegenerative diseases.

Figure 1

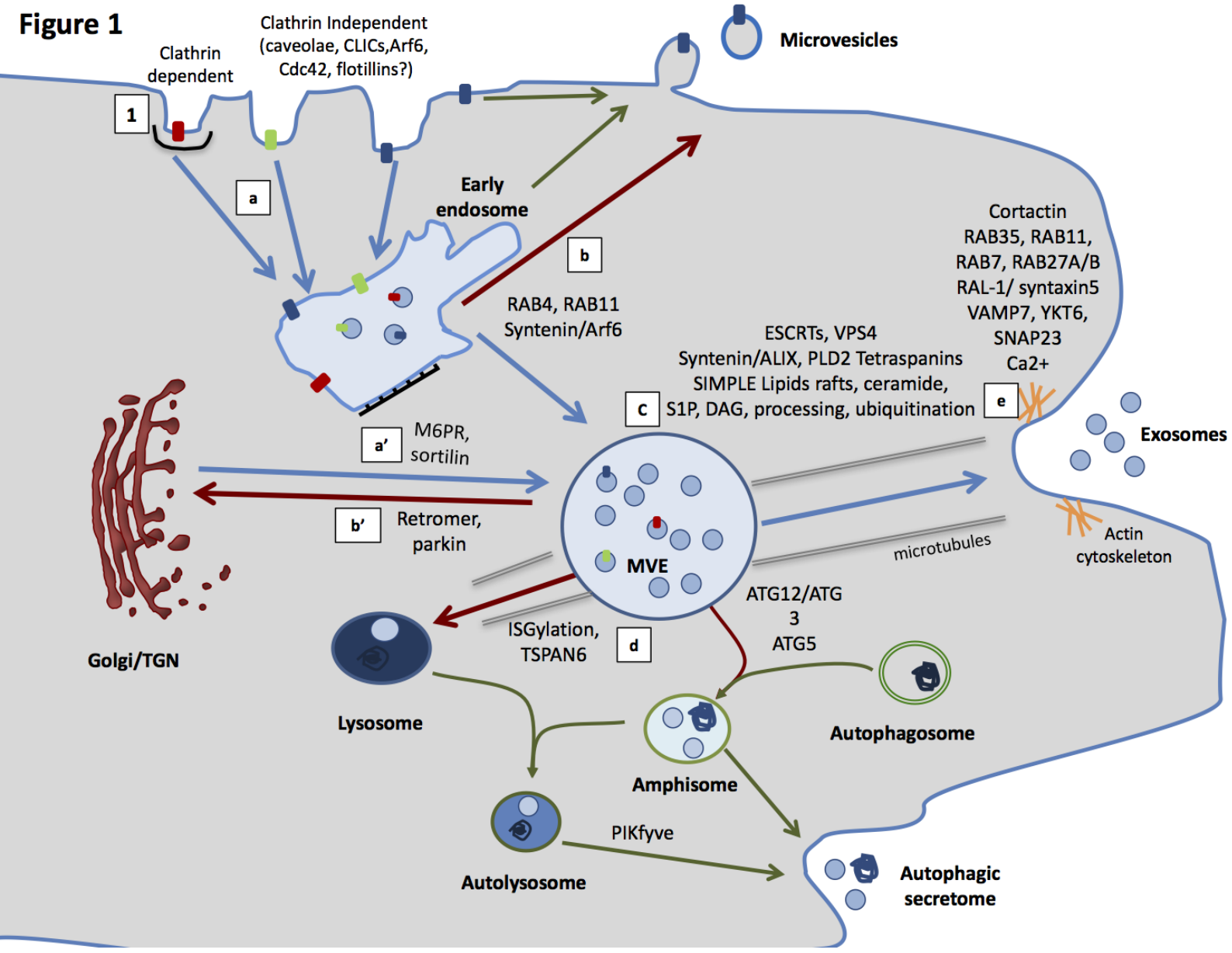

Clathrin dependent
Clathrin Independent (caveolae, CLICs,Arf6, Cdc42, flotillins?)
Microvesicles
1
a
Early endosome
b
RAB4, RAB11 Syntenin/Arf6
Cortactin RAB35, RAB11, RAB7, RAB27A/B RAL-1/ syntaxin5 VAMP7, YKT6, SNAP23 Ca2+
ESCRTs, VPS4 Syntenin/ALIX, PLD2 Tetraspanins SIMPLE Lipids rafts, ceramide, S1P, DAG, processing, ubiquitination
C
e
Exosomes
a'
M6PR, sortilin
b'
Retromer, parkin
MVE
microtubules
Actin cytoskeleton
Golgi/TGN
ISGylation, TSPAN6
d
ATG12/ATG 3 ATG5
Lysosome
Autophagosome
Amphisome
PIKfyve
Autolysosome
Autophagic secretome

Figure 2

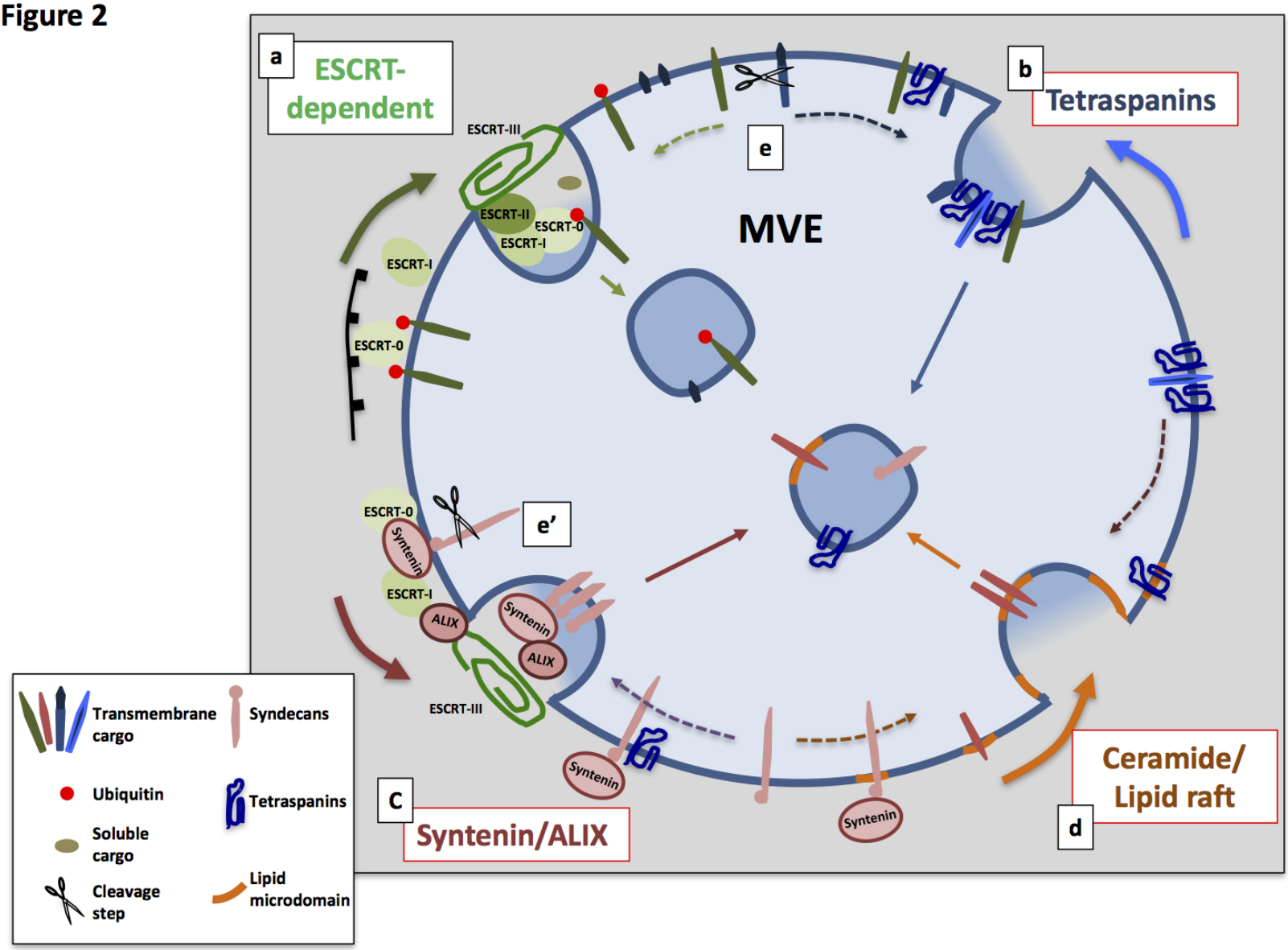
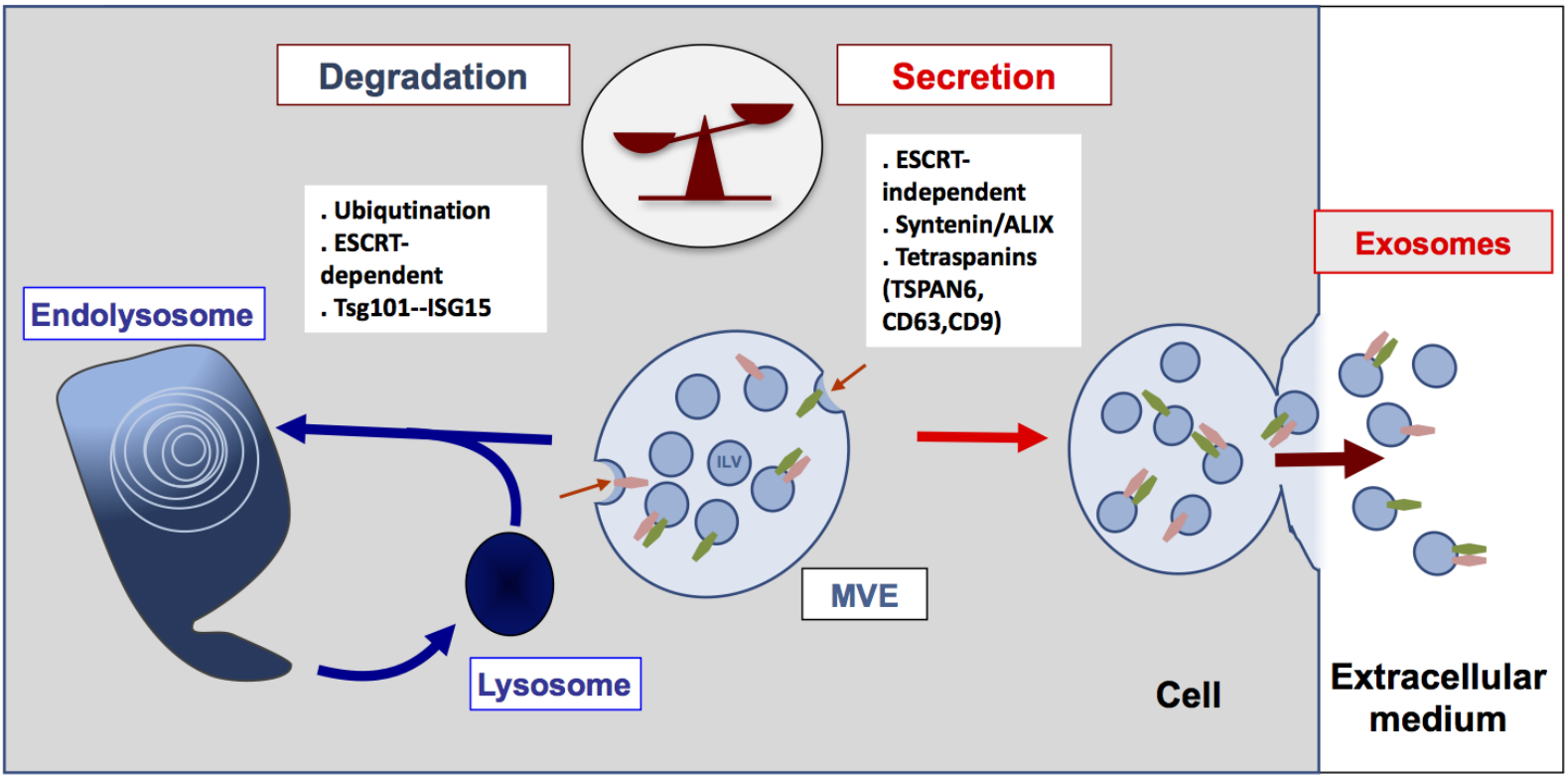

a
ESCRT-dependent
b
Tetraspanins
e
MVE
ESCRT-III
ESCRT-II
ESCRT-0
ESCRT-I
ESCRT-I
ESCRT-0
ESCRT-0
Syntenin
e'
ESCRT-I
ALIX
Syntenin
ALIX
ESCRT-III
Syntenin
Syntenin
C
Syntenin/ALIX
d
Ceramide/ Lipid raft
Transmembrane cargo
Syndecans
Ubiquitin
Tetraspanins
Soluble cargo
Cleavage step
Lipid microdomain

Figure 3


Degradation
Secretion
. Ubiqutination
. ESCRT-dependent
. Tsg101--ISG15
. ESCRT-independent
. Syntenin/ALIX
. Tetraspanins (TSPAN6, CD63,CD9)
Exosomes
Endolysosome
ILV
MVE
Lysosome
Cell
Extracellular medium