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Review

Understanding extracellular vesicle diversity – current status

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Abstract

Introduction: Extracellular vesicles (EVs) represent an important mode of intercellular communication. There is now a growing awareness that predominant EV subtypes; exosomes from endosomal origin, and shed microvesicles from plasma membrane budding, can be further stratified into distinct subtypes, however specific approaches in their isolation and markers that allow them to be discriminated are lacking.

Areas covered: Knowledge about these distinct EV subpopulations is important including the regulation of composition, release, targeting/localisation, uptake and function. This review discusses the mechanisms of distinct EV biogenesis and release, defining select EV classes (and subpopulations), which will be crucial for development of EV-based functions and clinical applications. We review the dynamics of cargo sorting leading to the mechanisms of EV heterogeneity, their mechanisms of formation, intracellular trafficking pathways, and provide an uptake about biochemical/functional differences. With advances in purification strategies and proteomic-based quantitation, allows significant benefit in accurately describing differences in EV protein cargo composition and modification.

Expert commentary: The advent of quantitative mass spectrometry (MS)-based proteomics, in conjunction with advances in molecular cell biology, and EV purification strategies, has contributed significantly to our improved characterisation and understanding of the molecular composition and functionality of these distinct EV subpopulations.

Keywords: extracellular vesicles, exosomes, shed microvesicles, proteomics, biogenesis, endosomal, trafficking, membrane

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1. Introduction

Cells continuously secrete extracellular vesicles (EVs) to the extracellular environment. There is increasing evidence that these secreted vesicles play important roles in numerous aspects of biology (e.g. intercellular vesicle trafficking, immunity, development, neurobiology and microbiology), contribute to many human pathologies (e.g. cancer, neurodegenerative disorders and HIV/AIDS) and have significant biotechnological potential. EVs facilitate the horizontal transfer of select proteins, lipids, RNA species (e.g., mRNA, miRNA, ncRNA) and single-/double-stranded DNA fragments between cells. EV uptake by recipient cells can reprogram signalling pathways to modulate the function and activity of the target cell [1-6]. Secretion and exchange of EVs occurs in all cell types and is fundamental in many different contexts and pathologies [7], including: blood pro- and anti-coagulation [8], innate/acquired immunity and immunomodulation [9, 10], stem cell differentiation [11, 12], tissue regeneration and angiogenesis [13], autophagy [14], embryo implantation [15], reproductive biology [16], placental physiology [17, 18], and tumour invasion and metastasis [19-22].

The origin, nature, morphology, size, and content of these vesicles are diverse and represent a novel signalling paradigm [23]. Despite diverse terminologies in the literature to define and identify EVs and debatable nomenclature [24], two main classes of EVs can be identified: shed microvesicles and exosomes [25, 26]. Shed microvesicles (sMVs, ~100-1,500 nm) are generated by outward budding and fission of the plasma membrane (PM) and the subsequent release of these vesicles into the extracellular space [27]. Exosomes (30-150 nm) originate as intraluminal vesicles (ILVs) via inward budding of the limiting membrane of maturing endosomes, referred to as multivesicular bodies (MVBs), which can fuse with the PM and release ILVs as exosomes into the extracellular environment [28]. Although an increasing number of studies have investigated the roles of EVs in cell–cell communication, understanding the distinct mechanisms involved in their biogenesis, and the diverse heterogeneity of EVs and their subtypes remains in question.

Recently, the presence of heterogeneous populations of EVs has been demonstrated, highlighting the need to address clear definition as to the number of EV subtypes, their mechanisms of formation and regulation, and how their biophysical properties and functionalities might differ. Similarities in biochemical and biophysical characteristics and molecular properties, including size/diameter, density, surface marker expression, composition, and membrane orientation of these EV subtypes, presents challenges with their

effective separation and represents a major challenge to the EV field [29]. Critical insight into the biogenesis, generation and secretion of exosomes [30] and sMVs [27] can be found in more detailed reviews. There is now a growing awareness that these EV classes can be stratified into distinct subtypes, however specific approaches in their definitive isolation, markers that allow them to be discriminated, and functional insights into their biology are limited. EV heterogeneity affects not only luminal cargoes but also the EV membranes. Heterogeneity is not a property specific to exosomes; it has also been demonstrated for sMVs. In this review we focus on recent advances in understanding the complexity of distinct EV classes, namely their biogenesis and mechanisms of composition, trafficking, release, and uptake, and provide an uptake on what is known about biochemical and functional differences. For both exosome and sMVs subtypes, their biology, composition and function result from the coordination of multiple intracellular molecular mechanisms. Comprehensive deciphering of the composition and functional diversity of EV subtypes needs to be addressed if we are to harness their potential for therapeutic use.

2. Categorisation of EV classes and respective subtypes

Typically, cells release two predominant EV classes: exosomes and sMVs [31]. An original report demonstrated that activated platelets release these two EV classes, demonstrating select differences in their particle size, marker (protein/membrane) expression and function (factor X and prothrombin binding capacity) [8]. Exosomes are formed by invagination of early endosome (multivesicular bodies, MVBs) and subsequent inward budding of the limiting membrane of MVBs to form intraluminal vesicles (ILVs). MVBs can either fuse with lysosomes and their contents degraded or alternatively, MVBs can traffic to, and fuse with, the PM [32, 33], where their contents (now termed exosomes) are released into the extracellular milieu [34]. Specific characteristics associated with exosomes include composition (bilipidic layer), size (30-150 nm diameter), density (1.09-1.13 g/mL) and protein content, including endosome-derived (endosomal sorting complex required for transport proteins; ESCRTs, such as Alix and Tsg101), sorting- and trafficking-related (endosomal Rab GTPases) and cell membrane-derived (tetraspanins CD63, CD81 and CD82) [35, 36]. Exosomes also contain various lipids and lipid-raft-associated proteins originating either at the PM or from early/late endosome compartments, including cholesterol, sphingomyelin, and flotillins [34, 37-39] (reviewed [40]). Exosomes also transfer select mRNAs and microRNAs (miRs) to neighbouring cells for translation [41, 42], and more recently shown to contain [43] and transfer DNA [44]. As will be discussed, specialised mechanisms act to ensure specific composition which will define the communication between exosome-

producing and target cell. As such, exosomes are known to adopt distinct roles dependent on their cellular origin. For example, modulating immune function [9, 10], stem cell differentiation [11], inflammatory responses [45], angiogenesis [46], lymphogenesis [47], cell migration [48], cell proliferation [49], immune suppression [50], invasion [19], epithelial-mesenchymal transition [51, 52] and metastasis [20]. Further, tumour-derived exosomes have been shown to prime distant organs towards a conducive microenvironment (pre-metastatic niche) [48, 53-62] to mediate survival and outgrowth of seeding tumour cells (metastatic niche) [20, 63], and facilitate transfer of metastatic capacity [21, 22] (reviewed [64]).

Shed microvesicles (sMVs, also known as microparticles and ectosomes) constitute a class of EVs with a heterogenous size range (100 to ~1,500 nm) [25, 37, 65-67] and generated by direct outward budding from the PM, involving cortical actin reorganization and subsequent outward protrusion of PM domains and detachment [68-70]. Compared to exosomes, sMVs are enriched in phosphatidylserine, cholesterol-rich/ specialized cell membrane microdomains (lipid-rafts), cell-lineage markers, and cell-surface receptors (review [25]). Unlike exosomes, the membrane composition closely resembles that of the parental cell [68]. Functionally, sMVs have been demonstrated to play a role in multi-drug resistance [71], immunosuppression [72, 73], evasion of immune surveillance [74], and the development of cancer [75]; cancer stem cell-derived sMVs have been shown to be implicated in pre-metastatic niche formation [76]. In contrast to exosomes, they exhibit a more diverse and higher buoyant density (1.09-1.19 g/mL), and differ in their protein content, for example, sMVs are rich in proteins associated with microtubule/cortical actin/cytoskeleton networks (Rac GTPase activating protein Racgap1), and effector components such as GTP-binding protein ADP-ribosylation factor 6 (Arf6) and its effector phospholipase PLD2, acid sphingomyelinase (A-SMase) activity, and specific components of the ESCRT family, including ESCRT-I [39, 65, 67, 77, 78]. Like exosomes, horizontal DNA/RNA transfer has been attributed to sMVs, thereby affecting gene expression and phenotype in recipient cells [79]. Their formation and release is reported to be highly regulated - the type and content dependent on the initial effector signal [80]. The molecular machinery for regulating outward budding of the PM and release has been shown to involve Arf6, Rac, RhoA, Cdc42, acid sphingomyelinase activity, and ESCRT components [25, 65, 77, 81, 82].

Apoptotic bodies, defined as large particles (~0.5–4 μm) containing cytoplasmic organelles and fragmented nuclei [83], are produced by cells undergoing programmed cell death, leading to budding and finally disintegration of the cell PM with partitioning of the cellular content in different membrane-enclosed vesicles [83], including apoptopodia formation [84].

Some studies have reported a communication and biological function for these vesicles, including genetic reprogramming [85, 86]. Like sMVs, expression of phosphatidylserine on the membrane surface is a key characteristic of apoptotic bodies [87]. Other vesicle types such as migrasomes [88], non-membranous nanoparticles known as exomeres [89], and autophagosomes [90], will not be covered in this review.

Originally, exosomes were thought to represent a homogenous class of EVs [91]. However, the heterogeneous nature of exosomes and the technical limitations in efficiently separating exosomal subpopulations have hindered the characterization of their molecular composition and biogenesis. Accumulating evidence from *in vitro* studies using cells grown in culture and *ex vivo* body fluids indicates the existence of distinct subtypes of exosomes (**Table 1**). Recent studies have revealed that different subtypes of exosomes are released from the apical and basolateral surfaces of highly-polarised human cancer cells [92]; these exosome subtypes are biochemically distinct from sMVs and one another based upon quantitative proteomics and genomics approaches [92-94]. Willms and colleagues further characterised subpopulations of cell-derived exosomes with distinct molecular composition and biological properties [95]. Subtypes of exosomes have been shown to differ based upon biogenesis/formation [96], lipid composition [97], and presence of stereotypic surface markers such as CD9 [98], CD63 [99, 100], CD133 [99], and GPA33 [92]. Alterations in ESCRT machinery (intracellular endosomal sorting components) have indicated the formation of distinct and heterogeneous exosomes in size and composition [101]. Recently, subpopulations of exosomes from a single-cell model separated by density-gradient centrifugation indicate diverse morphology based on multiple electron microscopy techniques [102]. Palma *et al*. showed differential packaging of miRNAs and subsequent release of distinct subpopulations of exosome-like vesicles by cancerous cells as compared to normal cells [103]. Further, technologies such as asymmetric flow field-flow fractionation [89] have the capacity to separate distinct exosome subtypes based on their density and hydrodynamic properties by perpendicular flow, and demonstrate distinct biophysical (size) and molecular properties (proteomic profiling, biodistribution localisation patterns) of each subtype. Further experimentation is required to establish whether altering exosome subtypes are functionally distinct.

Interestingly, distinct subtypes of sMVs with differing biochemical properties have been recently demonstrated; different biophysical properties including their capacity to float at buoyant densities in the range of 1.09-1.19 g/mL (*DWG/RJS, unpublished observations*). Further, sMV subpopulations with different buoyant densities – low density (1.09 g/mL) and high density (1.12 g/mL) - have been isolated by density (iodixanol) gradient fractionation

[104], indicating likely heterogeneous groups containing distinct cargo within the total microvesicle population [105]. Collectively, these studies raise several questions as to the nature of the underlying mechanisms responsible for their selective packaging, biogenesis, and biological significance of these microvesicle subpopulations. More recently, vesicles isolated from conditioned cell medium and blood plasma by ultracentrifugation were aligned into two distinct populations by bottom-up density gradient fractionation [95]. Both EV populations showed a different protein and nucleic acid composition, which correlated with a distinct biological effect on recipient cells [95]. Overall, EV subtypes (exosomes and sMVs) are comprised of a distinct repertoire of molecules [25, 78]. Importantly, there is a pressing need in the EV field to identify specific vesicle surface proteins in order to define EV class/subtypes – this information is critical to perform interlaboratory comparison and a better understanding of EV characterisation and functionality. Recently, we have investigated exosome membrane surface proteins (surfaceome) using a combination of sodium carbonate/detergent fractionation and proteinase K proteolytic digestion, in conjunction with proteome analysis, to identify integral and peripheral membrane proteins, RNA-binding proteins, and outer exosomal membrane surface proteins (*DWG/RJS, unpublished observations*). Characterisation of the surface composition of distinct EV populations is highlighted by a recent updated categorization of EVs based on enrichment of tetraspanins, one of the most commonly found protein class in EVs [104].

3. Mechanisms of distinct EV biogenesis

As depicted in **Figure 1**, exosomes and sMVs exhibit different biogenesis pathways. Exosomes originate via the endosome trafficking pathway; comprised of highly dynamic membrane compartments involved in the internalization of extracellular ligands or cellular components, their recycling to the PM, and/or their degradation [106, 107]. Early endosomes mature into late endosomes [32, 107], and during this process, accumulate ILVs in their lumen. The ILVs that are formed by inward budding of the early endosomal membrane sequester proteins, lipids, and cytosol that are specifically sorted. In most cells, the main fate of the MVB is to fuse with lysosomes [108], ensuring the degradation of their content. The process depends on surface proteins (such as HD-PTP, the HOP complex, and the GTPase Rab7) and on the assembly of a membrane-fusion soluble NSF attachment protein receptor (SNARE) system including Vamp7, Vti1b, syntaxin 7 and 8 [109-112]. However, organelles with MVB hallmarks; bearing the tetraspanin CD63, lysosomal-associated membrane proteins Lamp1 and Lamp2, and other molecules that are generally present in late endosomes (for example, MHC class II in antigen-presenting cells), can also fuse with the PM, whereupon the MVB limiting membrane integrates with the endosomal recycling system

and their ILV contents are released (now referred to as exosomes) [113, 114]. Involvement of both ESCRT-dependent (ESCRTs-0, -I, -II, and -III, syntenin-syndecan [101, 115-119]) and ESCRT-independent (neutral sphingomyelinase 2 (nSMase2)/ceramide formation [120], ceramide [121], sphyngosine-1-phosphate (S1P) [122], ARF6/PLD2 [77], and tetraspanin-dependent [123, 124]) drivers of MVB/ILV exosome formation have been demonstrated. Involvement of syntenin and syndecans in ESCRT-dependent processes have been highlighted, where syndecans interact with syntenin, which, in turn, interacts with CD63 and Alix through LYPX(n)L motifs [118]. Silencing of syntenin or syndecan decreases the number of released exosomes and reduces exosomal accumulation of Alix, Hsp70 and CD63, although no effect on flotillin-positive exosome formation. Exosome secretion is also affected by the silencing of the ESCRT-III component charged multivesicular body protein 2A (CHMP2A) [118]. Further, the small integral membrane protein of the lysosome/late endosome (SIMPLE) has been shown to play a role in exosome secretion [119]; SIMPLE is present in the ILVs of MVBs and in exosomes, and its overexpression increases exosome release and exosomal accumulation of Alix/CD63, although no effect in flotillin secretion. Existence of ESCRT-dependent and -independent mechanisms for the loading of select cargo in exosomes is not necessarily contradictory, but rather suggests the existence of specialized mechanisms to control the selective sorting of cargo into these vesicles, and therefore the presence of heterogeneous populations of MVBs and exosomes.

Members of the Rab GTPase family (including Rab 5/7/11/27/35) have been shown to modulate exosome trafficking and thought to act on different MVBs along these different endocytic pathways [125-128]. Further, V-ATPase was shown to be a key regulator of both cholesterol trafficking and endosome fate, with a significant increase in PM-associated exosomes when V-ATPase is inhibited [129]. The microtubule and cytoskeletal network has been recognised to regulate intracellular organization and transport MVEs, in coordination with molecular motors, to the site of release (reviewed [130]). For example, RAB27A and RAB27B (together with effectors, SYTL4 and EXPH5) act in the docking of MVEs to the PM in order to promote their fusion [128] and this mechanism requires the rearrangement of the actin cytoskeleton [131]. Further, RAB27A/B has been shown to facilitate the docking of MVBs to the PM, with reduction in exosome secretion after RAB27A silencing; this strategy is now commonly used as a way of modulating exosome secretion [132, 133]. Further, it is well known that specific lipid components, e.g. cholesterol, sphingomyelin, phosphoinositides endow regions of the PM with preferential capability to bind SNAREs and MVEs [134]. This suggests that the composition of the limiting membranes of MVEs may modulate their target location by acting on the motility of MVEs. However, not much is known regarding the fusogenic machinery implicated in exosome release. Recently, the PM-

associated protein tetherin has been shown as a exosomal tether, causing PM pooling of exosomes as imaged by fluorescence microscopy and correlative light/cryo-immuno/scanning electron microscopy [135]. Phosphorylated SNAP23 has been shown to enable exosome release [136, 137], in addition to MAP kinase PMK-1 [138], the morphogen Bmp [3], and Rab35 involved in a parallel recycling pathway to Rab11 and assists to traffic endosomes to the PM [139]. It is not known whether each of these pathways and tethering components acts on different MVBs or, concomitantly, on the same MVB. Future studies employing super-resolution microscopy and targeted molecular biology are needed to address spatial and temporal regulation of MVBs to the PM for exosome secretion. For example, optical tweezers have been shown to allow manipulation and visualization of individual exosomes from a subset lacking CD63 expression at the surface of recipient cells [140]. Alternative mechanisms of exosome release involve membrane fusion using a specific combination of soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) including vesicular SNAREs (v-SNAREs) localized on MVBs; these interact with target SNAREs (t-SNAREs) on the intracellular PM [141-143], release of cytoplasmic Ca^{2+} [144], inflammatory response (IFN-γ) [145], and cytoplasmic tyrosine kinase SRC [146] (reviewed [30]).

In contrast, the biogenesis of sMVs occurs by outward budding of select PM domains and regulated by a distinct set of molecular events including activation of AKT and acidic sphingomyelinase intracellular calcium flux variations, and enzymes involved in membrane phospholipid asymmetry [25, 78, 147-150]). The mechanisms involved in the biogenesis of sMVs are still being revealed [27]. Recent studies have suggested Rac, RhoA, Cdc42 in sMV biogenesis, sorting and release [81, 82], with reports highlighting the role of contractile proteins, phosphorylated MLCK2; a kinase that activates myosin II, allowing for contraction of the actin cytoskeleton [65], and MYO1A in sMV biogenesis [151]. ESCRT machinery has also been implicated in the sorting of protein cargo into microvesicles; Vps4 is involved in vesicles enriched in arrestin-domain-containing proteins (ARRDC) [152], with ubiquitination of ARRDC important for the secretion of ARRDC-bearing microvesicles [152]. Notably, during sMV biogenesis, vesicles typically retain select surface proteins and lipids and relocate phosphatidylserine on the extracellular side of the outer membrane [153]. Moreover, other phospholipids, such as unsaturated phosphatidylcholine and phosphatidylethanolamine, are not abundant in the PM domains are segregated during their formation [148].

Differences in the formation of EVs could affect their composition between distinct subtypes. Differential packaging of miRNAs and release of distinct subpopulations of exosome-like

vesicles by cancer cells has been demonstrated [154]. Recently, neuroblastoma cells secrete distinct populations of exosomes carrying different cargoes (specifically amyloid precursor protein-positive exosomes and its catabolites, in contrast to CD63-positive exosomes) and targeting specific cell types, including dendrites and glial cells [140]. Such dynamics suggest the selective release of subpopulations of exosomes possibly result from altered endocytic trafficking. Further studies have shown that cellular activation can modulate the dynamics of specific exosome populations [155]. The composition and quantity of secreted EVs is also dependent on *in vitro* culture conditions, where hypoxia can facilitate an increased release of CD63-positive vesicles [156] with a modified molecular composition and distinct effect on recipient cells [157]. Further, alterations in the EV protein composition is regulated by differing cell culture conditions; serum-containing and serum-starvation conditions [158]. Comparable observations have been shown for altered extracellular pH [159] and the presence of stress-inducing molecules (e.g. lipopolysaccharide, hydrogen peroxide) in general [160]. Further studies have suggested that subtypes of exosomes bind specifically to and are taken up only by distinct cell types for each subpopulation – indicating mechanisms which are selective in their target recognition and internalization [161]. For example, tumour-derived exosome subtypes expressing unique surface integrins can determine organotropism through selective targeting to either lung or liver sites [20]. Proteomic profiling revealed distinct exosomal integrin expression patterns, where integrins α6β4 and α6β1 were shown to be associated with lung metastasis, and integrin αvβ5 with liver metastasis. Selective inhibitory targeting of integrins α6β4 and αvβ5 decreased exosome uptake in target cells and tissues, as well as lung and liver metastasis, respectively [20]; therefore different exosome surface cargo components can facilitate different organ biodistribution patterns suggesting that they mediate the pleiotropic effects of cancer.

4. Exosome biogenesis complexity - different multivesicular endosomes and different multi-ILVs?

The endosomal pathway plays an important role in the sorting of membranes and cargo for ILVs. MVBs are much more dynamic and versatile than once recognized [106]. Endosomes function to limit and terminate signalling processes and function in signal propagation by facilitating the recruitment and integration of signalling cascades on the surface of endocytic vesicles [162-164]. Further, during cytokinesis endosomal sorting and trafficking into the midbody is probably needed to deliver key cargo required for lipid remodelling and PM dynamics [165, 166]. The ability of cells to upregulate MVB formation and degradative (lysosomal) capacity is exemplified by studies showing that growth factor stimulation can increase inward budding and MVB formation [167]. More recently, a link between endocytic

trafficking and epithelial polarity has been demonstrated [168, 169]. This is further supported by the involvement of ESCRT machinery to be a crucial determinant of cellular polarity [170]. Such studies are suggestive of a requirement to assemble populations of endosomes for defined cellular functions. Whether MVBs with different destinations exist as entirely separate entities is unclear. Identification of multiple populations of MVBs which differ in their protein and lipid composition have been described [167, 171, 172], indicating new markers of the endomembrane system. Moreover, MVBs and subclasses of α-granules ($CD63^+$) have been shown in studies of platelets and their activated states, as multivesicular compartments [173], and have suggested that these internal vesicles are released during platelet activation as exosomes [8] (reviewed [174]). Of interest, these MVBs were reported to be morphologically distinct, with their composition and presence of internal membrane vesicles [173] suggested to represent definitive developmental stage in α-granule maturation (i.e., ILV biogenesis). Further, reports that RISC (RNA-induced silencing complex) associates with endosomes, in addition to co-localization of AGO and Dicer, suggests that ESCRT-dependent sorting of RNA cargoes occurs in MVBs [175] and resultant exosomes [42, 176]. Exosome-associated RISCs may have the potential to be internalized by recipient cells, where they modulate gene expression and regulate function [42, 121, 160, 176-179].

The balance between recycling and targeting to MVEs further regulates the composition of MVEs and their subsequent trafficking (**Figure 2**). For example, syntenin in endosomes interacts with syndecans to support their recycling to the PM via a mechanism requiring production of phosphatidylinositol 4,5-bisphosphate PI(4,5)P2 and Arf6 [180], while syntenin, together with ESCRT accessory protein ALIX, can dispatch syndecans to ILVs [77, 118]. Further work in this area is needed to understand how endocytic vesicles between different endosomes are generated, at what point in the trafficking pathway do any distinctions develop, and how these distinct endosomal subtypes function and are specifically targeted throughout the endosomal pathway and to other organelles, including the PM and lysosome. Of note is the recent finding of Rab GTPases, RAB11 [144], RAB27A and RAB27B [128], and RAB35 [126] in targeting and docking MVBs at the PM to be released as exosomes (reviewed [125]).

The importance of MVBs in the regulation of multiple cellular functions and intercellular communication has facilitated interest in the mechanisms regulating ILV generation and the selective inclusion of sorted cargo. As such, ILV composition is not uniform; some MVE vesicles have been shown to be rich in select lipids, bis(monoacylglycero)phosphate (lysobisphosphatidic acid), yet exosomes do not contain this lipid [181]. In the context of exosome biology, the ESCRTs comprise the major machinery for MVB/ILV biogenesis [116].

Endosomal maturation (early-late endosomes) is regulated predominantly by the switching of the Rab GTPase RAB5 with RAB7 [182] (**Figure 2**). Further, the effective delivery of proteins to MVBs often relies on specific ubiquitination of cargo, recognition and sorting of ubiquitinated cargo to endosomal subdomains, and formation of ILVs (reviewed [32, 107]). Exosomes contain ubiquitinated proteins, implying their common origin with ILVs that carry ubiquitinated protein cargo for degradation [183]. However, ubiquitin-independent pathways, which generate highly specialized MVB-like organelles, use the same endosomal subdomains enriched in the same machinery that controls sorting of ubiquitin-dependent cargo into MVB/ILV pathway and release as exosomes [184]. Different tetraspanins have been proposed to have a role in ILV formation, including CD9 (knockout mice secrete fewer exosomes compared with wild-type mice [185]), CD81 [48], Tspan8 (alter exosomal protein and mRNA content [186]), and CD63 [187], and CD63-mediated ILV sorting LMP1 [188] and PMEL [123]. In addition ESCRT-independent MVB/ILV formation may also occur: these involve lipid-metabolizing enzymes such as nSMase2, which hydrolyses sphingomyelin to ceramide [120], and phospholipase D2, which hydrolyses phosphatidylcholine into phosphatidic acid [77] (Figure 2). Further, both ESCRT-dependent and -independent ILV formation can operate within the same MVB [187], with select processes regulating competing machineries generating subpopulations of ILVs which differ based on their size distribution and mechanism of formation. Given that ESCRT-dependent and -independent mechanisms have the capacity to generate heterogeneous population of ILVs in both size and cargo proteins [187], could then contribute to the heterogeneity in exosome subpopulations. It will be important to characterize further the molecular distinctions that categorize this potential exosome donor population(s) through the endosomal pathway, and the mechanisms by which potential luminal cargo is sorted into select MVBs and ILV populations. Recently, Jackson *et al.* [189] demonstrated that both exosomes and sMVs can be selectively enriched involving sequential polyethylene glycol precipitation and adsorption to immobilized lectin concanavalin A. In this study, inhibition of the ESCRT component ATPase VPS4, cause a dramatic reduction in release of both tetraspanins CD9 and CD63, as well as syntenin, a common EV component, and microRNAs, miR-92a and miR-150. Importantly, inhibition was further attributed to a decrease in the number of released EVs, as would be expected for a block of ESCRT-mediated vesicle budding. Interestingly, the study also highlighted that CD63 and CD9 are enriched in separable populations of EVs that are both sensitive to VPS4 inhibition, indicating that different EV subtypes are regulated by various ESCRTs. Further, the involvement of Arf6 in both exosome [77] and shed microvesicle [65] biogenesis, may control the balance between the generation of both subpopulations of EVs. These studies [77, 128, 189] support the notion of different MVB subsets can reflect distinct exosome types, and provide the availability of new molecular

markers for further insights to be gathered to dissect the heterogeneous molecular mechanisms of endosomal trafficking and EV composition.

5. Organisation at the plasma membrane – influencing microvesicle biogenesis

Formation and release of sMVs is a dynamic and physiological process that involves assembly of heterogeneous populations of membrane-enclosed vesicles [68]. It is well established that not all plasma-membrane proteins and lipids are incorporated into microvesicles [65]. Further, phosphatidylserine is relocated to the outer membrane leaflet, where microvesicle budding occurs, while the topology of membrane proteins remains intact [190, 191]. While similar to events associated with viral budding, including membrane curvature and asymmetry [192], organisation of specific locations throughout the PM has been shown to regulate the origin of sMVs.

Membrane proteins and lipids are often distributed in select regions on the cell surface. These regions are often assumed to be membrane domains, arising from specific molecular associations. Statistical simulations [193] suggest that membrane patchiness may result from a combination of vesicle trafficking and dynamic barriers to lateral mobility. When vesicle trafficking and endocytosis is inhibited, patches of integral membrane proteins and lipids on the cell surface increase, while their intensities decrease [194] – indicating a transient association between vesicle trafficking and cell surface membrane distribution. It is of note that in addition to their importance in vesicle trafficking, the budding process of sMVs appears to occur at specific sites on the PM and is designed to release select cellular components into the surrounding environment, particularly cargo involved in cell-matrix interactions and matrix degradation [195, 196]. Functional proteomic analysis of lipid rafts using quantitative high-resolution MS and cholesterol-disrupting drug treatments, revealed correlation with known signalling factors and vesicle trafficking proteins, including SNAP23 and flotillin-1 [197]. The origin of sMVs has been shown to occur from various origins on the PM, including at microvillar protrusions of intestinal epithelial cells [151] and from cells engineered to overexpress hyaluronan synthase [198], and from cilia [199].

Understanding the detailed organization of cellular membranes remains a complex issue [200]. Select cholesterol- and sphingolipid-enriched domains, including lipid rafts, modulate PM organization, cell signalling, protein–protein interactions and other important biological processes. Aminophospholipid translocases including flippases, floppases, and scramblases regulate the movement of phospholipids, transbilayer lipid asymmetry, and organisation of

PM phospholipids [201]. Importantly, these proteins regulate the appropriate membrane topology through phospholipid movement and phosphatidylserine subcellular distribution [202]. Other protein classes including lipid transfer proteins are proposed to be involved in the localization of specific lipid species potentially by mediating transport of lipids at membrane contact sites [203]. Thus, the interplay between intracellular lipid distribution and organisation is crucial for the regulation of vesicle budding, vesicle fission and vesicle fusion. Further, cytoskeletal rearrangements are controlled by the Rho family of small GTPases and their downstream signalling cascades, result in distinct types of actin-rich invaginations or protrusions such as filopodia, lamellipodia, invadopodia, podosomes, phagocytic cups, uropods, PM budding (reviewed [204]). Budding is initiated by a combination of events (including intracellular and extracellular responses involving contractile networks [205]) that involve local disruption of membrane–actin cortex interactions, involving Arp2/3 complex assembly factors, septin members, Rho, ROCK, myosin, and various select components of the cytoskeleton network [206, 207] (reviewed [208]). Recently, select protein markers for sMVs have been discussed based on enrichment strategies and quantitative proteomics and cellular origin [39, 67, 78, 92, 103, 104, 209, 210], including members of the septin family, kinesin-like protein (KIF23), exportin-2/ chromosome segregation like-1 protein (CSE1L/CAS), v-SNARE components VAMP3/7, Rac GTPase-activating protein 1 (RACGAP1), actinin-4 (ACTN4) and mitofilin (IMMT). The molecular mechanisms leading to association of such proteins to sMVs needs further investigation, but such profiling experiments suggest that extracellular matrix, lipid-raft, cytoskeleton network, and microtubule motor proteins represent a particular category of sMV-associated components. However, what defines select sites of formation and release, and how cargo's including soluble proteins or nucleic acids are targeted to microvesicles remain limited (reviewed [27]). Importantly, sorting of proteins into sMVs has been suggested to involve PM protein oligomerization (i.e., N-terminal acylation tag) [211]. Several key examples for selective cargo recruitment at the budding sMV include Arf6, Rab22A, and v-SNARE/VAMP3. Arf6-regulated endosomal trafficking has been shown to selectively recruit and incorporate protein cargo in tumour-derived microvesicles [65], while Rab22A co-localizes to shedding microvesicles, increases their release, and selectively recruits cargo proteins utilized for formation under hypoxic conditions [212]. Recently, vesicle-associated membrane protein family member VAMP3 has been shown to deliver microvesicle cargo such as MT1-MMP, to regions of high PM budding [209]. This process highlights the role of VAMP3 for the association of MT1-MMP to enable CD9-mediated delivery to the cell surface [213]. Therefore, understanding the precise mechanisms for cargo sorting, trafficking, membrane organisation and contraction, and the pinching events that coordinate the release of subtypes of sMVs remain to be further investigated. There are multiple signalling pathways

that are now thought to regulate microvesicle formation and highlight a potential role for extracellular signalling factors, including growth factors (TNF-α), in cell activation and biogenesis and release of distinct populations of protein and RNA cargo [214, 215].

6. The cargo complexity – understanding the molecular composition of EVs

EVs containing disparate molecular cargo such as protein, mRNA, miRNA, DNA and lipids, can be directly internalized by target cells, and induce functional change [2, 43, 216, 217]. However, various mechanisms of EV cargo transfer exist and how the importance of EV membrane/surface components in regulating target recognition remain important questions.

Recently, exosomes have been highlighted to carry Wnts on their surface to induce Wnt signalling activity in target cells *in vivo* and *ex vivo* [143]. Using immunogold labelling canonical and non-canonical Wnts were identified on extracellular EVs with the morphological and biochemical characteristics of exosomes. Moreover, α-synuclein has been shown using immunogold staining to be both outside and inside exosomes from primary sympathetic neuronal cells [218]. Further, using select proteases to shave off surface proteins of exosomes [219], resulted in a reduction in their transfer and cellular uptake capacity [220], suggesting proteins on the surface of exosomes and target cells act as mediators to, in part, facilitate recognition/interaction. Exosomal LOXL2 has been shown located on the exterior of exosomes, where exosomes were treated with proteinase K in the presence of detergent TX-100 to permeabilize exosome membranes [221], with control membrane/cytosolic proteins to monitor the integrity of the exosomes and confirmed by electron microscopy. Further, we have demonstrated that proteolytic digestion (proteinase K) is capable of cleaving surface-exposed proteins while maintaining vesicle integrity with label-free mass spectrometry proteomic profiling to reveal the exosome surfaceome (*DWG/RJS, unpublished observations*). It is anticipated that such “EV surface” experiments will reveal much about the luminal contents and surface (targeting) constituents of EVs. Further, whether RNA species are selectively packaged as integral components of EVs [222] and not just bound to the outer surface of the vesicle complexed with RNA binding and chaperone proteins (i.e., in the extracellular space) remain to be investigated further. It is important to consider that many studies have raised concern whether identified extracellular RNAs are associated with EVs or instead with RNA–protein complexes co-isolated with EVs. Importantly, we highlight surface-localised miRNA-loaded RNA binding proteins (RBPs) and RNA nucleoproteins (RNPs) associated with the exosome surfaceome (*DWG/RJS, unpublished observations*). The addition of RNase to EV pellets has been shown to reduce

the RNA content by ~7% [176, 223]. Whether RNAs are within the cytosolic lumen or associated with the EV membrane can be achieved by measuring flotation into density gradients and resistance to RNase digestion subsequent to protease treatment. This may provide an indication as to utilising different RNA isolation methods give extensive variation in exosomal RNA yield and species distribution patterns [224]

As discussed, EVs derived from cancerous cells have shown to carry a panel of known (e.g. CEA, GP100, HER2, melan-A, PSMA, melanoma-associated antigen D2 (11B6), glypican-1, BJ-HCC-24 tumour antigen, and mesothelin (CAK1 antigen)) [225, 226] and unknown tumour antigens. Importantly, EVs from different tumour cells have shown immune activity against not only syngeneic but also allogeneic tumour growth, indicating that tumour-derived exosomes may harbor select subsets of tumour antigens capable of inducing antigen-specific immune responses [227]. Therefore, tumour-derived EVs are a natural and novel source of tumour antigens which could provide alternative diagnostic circulating markers for mesothelioma and its progression but also may represent attractive tumour-specific therapeutic targets [9, 10, 226, 227]. Clearly, this is not only of interest from a diagnostic aspect but also makes tumour-derived EVs, which have shown to be more selective as free antigens [228, 229] and tumour lysate [230], an attractive candidate to evaluate as a cell-free vaccine.

EV-DNA has been shown to reflect the parental cell genomic DNA, because cancer cell mutations in BRAF, EGFR, KRAS, c-Myc, and p53 were detected in EV-DNA from melanoma and pancreatic cancer cells [43, 231, 232]. With respect to the presence of EV-DNA in EVs, the functional significance of this cargo remains unknown. A recent study has indicated that horizontal DNA gene transfer can occur by total EVs [233]. Such studies have revealed that DNA is not contained within the membrane-enclosed space of EVs, but is largely attached to the outer surface of EV [234]. The mechanisms of DNA packaging in/associated with EVs and their subtypes are poorly understood and whether DNA is packaged within the membrane-bound space of EV subpopulations remains unclear.

The specific mechanisms of RNA sorting into EVs are still being defined, with significant effort presently in understanding their mode of selection, localisation and interaction. Mechanisms of exosome sorting of RNA appear to be occurs before the budding process, when RNA molecules bind to raft-like regions of MVB membranes [235, 236], and independent of ESCRT and dependent on ceramide [237]. Also, hydrophobic modifications, lipid structures, and organisation of rafted membranes has been proposed as mechanisms for RNA binding and loading into exosomes [238]. Further, sorting of miRNAs into exosomes

has recently been shown to be directed by synaptotagmin-binding cytoplasmic RNA-interacting protein (SYNCRIP) [239]. Argonaute proteins bind mature miRNAs, with Argonaute 2 (Ago2)-associated miRNAs sorted into exosomes in a process regulated by KRAS signalling [240, 241]. RNA-binding protein (RBP) Y-box protein 1 (YBX1) has been shown to also facilitate sorting of a non–Ago2-bound miRNA (miR-223) into exosomes, highlighting a RBP-mediated exosome cargo sorting pathway [242]. Recently, the role of YBX1 in the sorting of highly abundant small ncRNA species, including tRNAs, Y-RNAs, and Vault-RNAs, including a unique post-transcriptional modification in the sorting of some RNA species into EVs, has been described [243]. Specific nucleotide motifs (EXOmotifs, GGAG motif) have been shown to regulate the loading of select miRNAs into exosomes. EXOmotifs are recognized and bound by the sumoylated ribonucleoprotein A2B1 (hnRNPA2B1) to control the sorting of RNAs into these vesicles [244]. Further, enrichment of 3'-adenylation and uridylation has been shown in cells and exosomes, respectively [245] - suggesting that post-transcriptional 3' uridylation may contribute to exosomal sorting/secretion of small noncoding RNAs. Limited molecular mechanisms which suggest select RNA loading into sMVs have been described; a zipcode-like 25 nucleotide sequence in the 3'-untranslated region of mRNAs, with variants of this sequence present in many mRNAs enriched in sMVs [246]. Of note, features of this sequence include a CUGCC core similarity presented on a stem-loop structure and a miRNA-binding site. Further research into determining the involvement of select RNA-binding proteins and RNA motifs to regulate select cargo sorting into EVs and their subtypes, in addition to whether distinct types of RNA (i.e., miRNAs and mRNAs) are sorted together, actively remains in question.

To understand distinct RNA profiles between distinct subtypes of EVs, Ji et al., [93] and Chen et al., [94] have performed miR profiling and transcriptome and long noncoding RNA (lncRNA) sequencing, respectively. Three distinct EV subtypes from human cancer cells were purified using a combination of immunoaffinity and ultracentrifugation, to investigate sMVs and two distinct exosome populations [93]. Using deep sequencing prominent and selectively-enriched signatures between each EV type were revealed, including star miRNAs, miR-19a/b-3p, miR-378a/c/d, and miR-577 and members of the let-7 and miR-8 families. This finding suggests miRNA biogenesis may be interlinked with endosomal/exosomal processing. With respect to transcriptome/lncRNA profiling [94], the authors demonstrate that 2,389 mRNAs, 317 pseudogene transcripts, 1,028 lncRNAs and 206 short non-coding RNAs selectively enriched in EVs, relative to parent cell. Interestingly, various splice-variant and missing proteins and RBPs and ribonucleoproteins (RNPs) were observed in these EV subtypes at the proteome level [92] with possible cognate RNAs

identified at the RNA level. Such studies provide unique insights into distinct protein, miRNA, and mRNA/lncRNA signatures within unique populations of EVs from a single cell origin. With respect to exosome subtype heterogeneity and the dynamics of miRNA load, the same cell source has been shown to exhibit a dramatic quantitative heterogeneity of its miRNA cargo [247]. This study revealed select stoichiometry of miRNAs and exosomes, suggesting that most individual exosomes do not carry biologically significant numbers of miRNAs and are, therefore, individually unlikely to be functional as vehicles for miRNA-based communication. These results indicate that current techniques and methods need to be developed to enrich the subpopulation of miRNA-rich exosomes, and functionally sufficient quantities of exosomal miRNAs need to be determined.

7. Defining homogenous EV types – methods of definitive isolation and purification

Several strategies have been used for EV subtype isolation and purification including differential ultracentrifugation, density gradient separation, affinity isolation capture, size exclusion and gel permeation chromatography, precipitation (i.e., sequential polyethylene glycol precipitation/lectin concanavalin A), and sequential ultrafiltration (**Table 2**) (reviewed [248]). Each approach is dependent on several key factors including the purity of EV subtype, yield, scalability, integrity requirement, time, cost, and suitability of the approach (i.e., availability of surface marker antigens for immunoaffinity isolation). Often, projects are directed towards extensive purification and fractionation to dissect EV subtype heterogeneity and advance understanding of EV characterisation, biology, formation, and function [249], while other studies require a rapid, reproducible approach for application of EV-based diagnostics and therapeutics [250]. It is important to apply stringent purification measures to elucidate the biophysical and biochemical properties of distinct subtypes of EVs [251, 252]. It is therefore clear that functionality and diagnostic/therapeutic use of EVs can only be truly defined once the range of EV sub-populations from a given source are isolated for complete and homogeneous analysis of constituent molecules and described in full. To define the functions of EVs *in vitro* and *in vivo*, it is necessary to the understand these vesicles themselves and therefore develop complementary methodologies to allow their efficient separation and targeted analysis [67, 189, 253]. Presently, we are still lacking separation techniques that can be used to definitely isolate and purify different EVs for downstream functional assays. With the functional implications being defined for EVs, it is vital to understand these vesicles themselves. A seminal challenge in the field is to establish methods and identify stereotypical markers that will allow for homogeneous discrimination

between exosomes, sMVs, and other EVs [78, 252, 254]. Affinity-based immunoisolation allows for select isolation of subtypes of EVs which differ based on extracellular antibody surface expression; however, concomitant co-isolation of the antibody/bait may perturb functional insights using this approach. Therefore, accuracy in purification and care in utilising appropriate controls are important aspect of analysing EV function. Collectively, this knowledge will enable identification of molecular tools to specifically isolate, modulate secretion, and potential function of a select EV subtype.

8. Proteomics and proteogenomics in EV biology

Proteins are critical bioactive constituents of EVs, and involved in regulatory functions associated with cargo sorting, trafficking, release and interaction/uptake [53, 255]. EVs typically display cell surface proteins derived from their cell of origin, which can be recognized by cell surface receptors (e.g., proteoglycans) and internalized by recipient cells [256], resulting in transfer of EV contents. EVs have demonstrated capability of mediating intercellular transfer of proteins causative for target cell function, including EGFRvIII [257], Met [54], mutant KRAS [255], CD147 [258], GPC1 [259], MIF [53], and select extracellular integrin cargo associated with tissue-specific targeting [20]. These seminal studies have described how EV-containing proteins are of fundamental importance in EV biology. Further, different post-translational modifications (PTMs) can regulate the sorting of proteins in exosomes, namely ubiquitination, sumoylation, phosphorylation, oxidation, ISGylation, and glycosylation [183]. Proteolytic processing of exosomal cargo proteins has also been demonstrated, including heparanase [260] and PMEL [123], to influence the sorting and trafficking of cargo proteins destined for exosomal release. Therefore, technologies which can further describe key differences in EV protein cargo composition and modification, monitor reprogramming of select target cells, and prioritize their involvement in various physiological and pathological processes, would be of significant benefit to the field of EV biology and cell communication.

Proteomics can be described as the large-scale study of the structure and function of proteins in complex biological samples – this discipline encompasses PTMs, protein spatio-temporal localization and, importantly, protein-protein interactions (referred at as the interactome). Mass spectrometry (MS) is a powerful technology for the quantitative identification of protein components of EV subtypes – information that is fundamental for understanding their biogenesis, function, as well as discovery of stereospecific protein markers that might allow EV subtype discrimination [39]. MS-based proteomics has assisted in defining EV surface proteins including EpCAM, CD24, CA-125, CA19-9, Met, GPA33,

EGFR, CLDN3, and heparan sulfate proteoglycans, shown to be significantly enriched in cancer EVs [54, 67, 256, 261-263]. There are currently two prevalent strategies for protein based quantitation, referred to as either label-free or differential labelling based approaches which have been applied to understanding EV composition and biology (reviewed [264]). In general, label-free methods have been favoured for quantitative proteomic studies due to their relative low cost, protein identification capacity, and high-throughput [265]. However, label-free strategies have several limitations, including dependence on accurate mass measurements and reproducible peptide retention times by liquid chromatography, which if not carefully controlled can introduce bias in peptide ion intensities [266]. By contrast, metabolic labelling strategies such as stable isotope labelling with amino acids in cell culture (SILAC) and ^{15}N-labeling can alleviate these issues, but they have limited applicability because they require specialized growth conditions contributing to longer development time, are not often amenable to *in vivo*/clinically-derived samples, and increased cost. Development of multiplexed quantitation via isobaric chemical tags (e.g., tandem mass tags (TMT) and isobaric tags for relative and absolute quantitation-iTRAQ), circumvent the limitations of both metabolic labelling and label-free strategies. Notably, these tags have identical chemical structure, yet have unique mass reporter ions that are used for MS/MS quantification. This strategy enables the multiplexing of all peptide sets prepared from different clinical samples to be combined into a single LC-MS/MS analysis and thus improves throughput and the breadth of coverage by avoiding missing values that are common in label-free based quantification. Due to the isobaric nature of the tags, all shared peptides from the combined samples exhibit the same biochemical properties (i.e., exact mass, and ionization efficiency and retention time). In EV biology, studies have employed various label-based approaches including SILAC [103, 267-269], multiplexed TMT labelling [270-272], and multiplexed iTRAQ labelling [273-276] (reviewed [264]).

In the field of EV research, it is becoming increasing important to apply stringent and targeted purification strategies to differentiate between EV subtypes; such approaches allow insight into their distinct molecular attributes (i.e., luminal or surface components) [251]. In fact, dissecting the heterogeneity of EV populations by approaches including differential ultracentrifugation, immuno-affinity capture, ultrafiltration and size-exclusion chromatography, polymer-based precipitation and microfluidics in an attempt to separate nanoparticle populations has proven daunting. Recently, asymmetric flow field-flow fractionation technology has been applied to separate discernible exosome subpopulations, and a distinct EV subtype (exomere), which differs in size and content from other reported

particles [89]. This approach further utilised proteomic profiling, glycomics, lipidomics and genomics to reveal additional distinct molecular signatures between exomeres and exosome subtypes; such approaches highlight the diversity of EVs and particles secreted by cells, and insights into marker expression of distinct subtypes of EVs. Recent proteome-wide studies involving RNA interactome capture and system-wide analysis of protein–RNA interactions have significantly increased the number of proteins implicated in RNA binding and uncovered hundreds of additional RBPs lacking conventional RBP domains [277]. Using high-throughput protein identification and characterization, other investigators have identified nucleoproteins in exosomes. Based on RNA electrophoretic mobility shift assay and affinity-based purification with LC-MS/MS analyses (label-free, LTQ-FT mass spectrometer), RBPs have recently been identified in exosomes capable of interacting with cell-RNA, cell-miRNA and esRNA [278]. Further application of understanding protein-protein and protein-RNA interactions, previously highlighted to be fundamental in the specificity of intracellular vesicular trafficking [279], will need to be focused towards EVs. Proteomic-based protein-protein interaction identification have become instrumental in biologically focused investigations for single or targeted proteins of interest [280].

Proteogenomics is an emerging field of biological research at the intersection of proteomics and genomics (reviewed [281, 282]). Proteogenomics can provide empirical evidence for the existence of proteins and protein variants, which can help delineate the set of protein-coding genes in the genome. Proteogenomics can be used to study the effect of genetic variations on the proteins they encode, providing a comprehensive understanding of genotype-phenotype relationships as proteins are more direct determinants of function. Importantly this area of research provides insight into the identification, analysis, and interaction between protein and RNA of EV subtypes [283]. As such, this emerging field provides key insights towards underlying mechanisms of EV biology and function, discover therapeutic targets, or generate biomarkers for diagnosis or therapeutic applications.

9. Expert commentary

The origin, nature, morphology, size, and content of EVs are diverse. There is now a growing awareness that predominant EV subtypes; exosomes (~30-150 nm vesicles) from endocytic origin and sMVs (~100-1500 nm vesicles) from PM budding, can be further stratified into distinct subtypes, however specific approaches in their isolation and markers that allow them to be discriminated are lacking.

Exosomes and sMVs function as a fundamental mode of intercellular communication and molecular transfer. These subcellular signalling entities have brought about a new paradigm in cell communication. However, despite the plethora of novel functions and roles of EVs and their subtypes, the field has been limited by methodologies and technologies to characterize and validate the identity, purity, and quantity of subpopulations of EVs. Further, EV heterogeneity affects not only luminal cargoes but also EV integral and peripheral membranes (i.e., surfaceome). Significant efforts are underway towards detailed characterization of heterogeneous EV subpopulations - defining context-dependent EV markers and function, where in-depth biophysical characterisation of EV subtypes and appropriate biological responses are crucial [254]. Different EV subpopulations from different cellular origins, or even the activation state of the cell-derived vesicle population, will produce a specific response on target cells. Specific responses, such as thermal and oxidative stress, can increase immunosuppressive exosome release from leukemia and lymphoma T and B cells [284] and reprogram the proteome and genome of exosomes that influence the resultant target cell [285, 286]. With increasing numbers of comparative proteomic studies of EV subtypes, this information will provide generation of new tools for the isolation of protein cargo within distinct EV types, and insights into context-dependent protein markers to define the types of EVs from cellular or biological fluids. Importantly, such information will provide new pathways and mechanisms to understand the biogenesis and regulation of EV subtypes.

This review has focused on recent knowledge in the areas of EV biogenesis and release, defining distinct types of EVs (and their subpopulations within), mechanisms of biogenesis, cargo selection, and understanding the cargo complexity of EV subpopulations. In addition, we highlight different trafficking pathways which may coordinate the formation and release of distinct EV subtypes including multi-vesicular endosomes, intraluminal vesicles, and organisation at the PM and how this may influence biogenesis of exosomes and microvesicles. Importantly, we focus on the need for comprehensive deciphering of the composition and functional diversity of EV subtypes for any patho/physiological systems in order for qualitative effects to be monitored, as suggested by the International Society for Extracellular Vesicles (ISEV) [287]. Advances in definitive isolation and purification of EVs, in combination with approaches for their characterisation (proteomics, genomics, and proteogenomics [39, 245-247, 288, 289]), and molecular biology (imaging, localisation, trafficking, expression, transfer, and manipulation [21, 290-292]), in addressing molecular mechanisms of EV biology.

10. **Five Year View**

The EV field remains an exciting and relatively underexplored area. During the next several years, important developments are expected to occur in the EV biology and technology, expanding the present knowledge of EVs and their application. Among these, as a key focus of this review, are the isolation and detailed characterization of heterogeneous EV subpopulations. Understanding how different sorting mechanisms determine the incorporation of specific molecules into exosomes or distinct vesicle subpopulations carrying different cargo is a fundamental area of active research.

For EV subpopulations, further sub classification and characterisation will occur with the recent advances in high-performance MS are up to the task of determining a comprehensive landscape of proteome and PTM-based proteome dynamics, and signalling pathways within EVs of vanishing small amounts of material. With the advent of orthogonal strategies for EV isolation, will lead to the identification of various EV and sub-EV markers. These data will not only provide a valuable resource for further mechanistic studies of individual proteins in EV biogenesis, trafficking, cargo sorting, and release. but also suggest additional players and regulatory mechanisms governing intra- and extracellular signalling. Intervention of mechanisms involved in target cell recognition and internalisation of EVs and recipient cells will provide key insights into the biological effects of EVs, and the control of target cell signalling pathways. Comparative analyses between transcriptomic and proteomic data will enable post-transcriptional regulation in this space to be further investigated. In cell signalling research, the combination of targeted enrichment methods and MS-based proteomics has provided fundamental advances and insights into understanding key mechanisms [293-296]. Increasingly, proteomics is using technologies to not only investigate protein level changes, but also the complex interactions of signalling molecules and networks [297]. Such approaches are now being applied to monitor and understand subtleties in dynamics of protein synthesis occurring uniquely within the actual cellular context [298]. Understanding the role of protein complexes within EVs will have significant biological implications for target cell selection and internalisation. Recent technological advances such as stabilised affinity MS [299] to preserve native interactions in EVs, are being applied to target proteomics and understanding complex protein cascades in EVs [300, 301]. Finally, the increased knowledge about the content, mechanisms of formation, transfer, and function of various EVs will provide important developments in the utility of EVs in preclinical, prognostic, and therapeutic applications. However, our understanding of the constituent and functional differences between EV types, their ability to be isolated, detected, or their half-life in biological samples remains limited. Developing applications in targeted MS establish the foundations for proteomic platforms that could be used for high-

throughput analysis, verification and validation. In particular, proteomics could be used for rapid quantitative analyses of EV protein panels, signalling pathways and pharmacokinetics. This could be further extended for use in multiplexed assays, drug discovery and clinical applications for EV biomarkers [302-305].

Key Issues

- New insights in the heterogeneity of EVs – defining standardized isolation protocols, global and cell-type specific markers of each subtype. Importantly, providing stereotypical markers that will allow for homogeneous discrimination between exosomes, shed microvesicles, and other EVs
- Does the relative importance of EV subpopulations depend on the tissue type in question and physiological context? How will the EV proteome and genomic landscape differ between EV types in different tissues, biofluids, and cells?
- Monitor and assess EV quantity – quantification approaches are diverse and often focused towards a particle size range
- International efforts to standardise nomenclature, reporting and centralizing knowledge are active and ongoing [29, 306].
- High resolution quantitative MS has been successfully applied to quantify thousands of PTM sites. These data provide a basis for directed functional analysis on single proteins as well as for 'systems-wide' studies.
- There are now applications of high resolution quantitative MS in elucidating global signalling networks and their dynamics in response to different cellular perturbations. Applications are currently underway for EV-focused studies and assessing functional transfer
- Bioinformatic analysis of proteomic data can provide insights into the nature and evolution of signalling networks and how these are regulated through EVs
- Characterising the processes that regulate the specific recruitment of proteins into EVs due to different physiological and pathological response is required. Understanding the pathways that regulate these mechanisms is also required
- EV heterogeneity affects not only luminal cargoes but also the EV membranes. This knowledge will further understand mechanisms of EV target and fusion with recipient cells – defining the interactome of EVs
- How do the membrane contexture of exosomes and other physicochemical configurations influence their target cell selectivity? Extending our understanding of distinct mechanisms that underpin cell-type specific EV recognition and entry into cells

- Defining factors influencing sorting of cargo into EV have been identified and experimental systems have been designed to address functional transfer of EV-RNAs in vitro and even in vivo. What is the suitability of different cargo-loading approaches for encapsulating components within/on EVs?
- Through affinity-based quantitative proteomics will allow specific proteins to be identified and subsequently investigated in EVs using molecular biology, for example, by interfering with their incorporation into developing exosomes, or modulating their capacity to target and transfer function

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* of interest

** of considerable interest

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References of Importance

(*=of importance, **= of considerable importance)

** Ref 64 (Xu 2016)

comprehensive overview of the properties, functions, implications in cancer biology, and challenges associated with exosome research.

* Ref 7 (Yanez-Mo, 2015)

Definitive resource for biological properties of extracellular vesicles and their physiological functions

* Ref 89 (Zhang 2018)

Defined distinct subtypes of extracellular vesicles, and a unique subtype nanoparticle, exomere

* Ref 104 (Kowal 2016)

Performed density-based fractionation and immuno-isolation to comprehensively characterization of heterogeneous populations of EV subtypes to reveal novel markers

* Ref 92 (Tauro 2013)

Sequential immunocapture and in-depth label-free-based mass spectrometry was used to identify distinct populations of exosomes (from apical and basolateral surfaces), which were different in biophysical characteristics and proteomic profiling to sMVs.

** Ref 94 (Chen 2016)

Parallel purification strategy to immune-isolate exosome subtypes and shed microvesicles from cancer cells, transcriptome and long noncoding RNA sequencing and in-depth characterisation to reveal EVs are biochemically distinct with unique RNA cargo.

* Ref 8 (Heijnen 1999)

An original report on platelet-derived EVs highlighting several distinct mechanisms resulting in vesicles of different sizes and architectures.

** Ref 25 (van Neil 2018)

Critical review providing insight into EV-generation machineries which act concomitantly and separately for the generation of distinct EV subtypes.

* Ref 128 (Ostrowski 2010)

Key study highlighting involvement of select Rab GTPases RAB27A and RAB27B32 and their respective effectors essential for exosome secretion. This and other such studies demonstrated direct regulation of GTPases on the potential priming of MVE secretion.

Table 1 **- Selection of exosome and shed microvesicle subtypes secreted from various cell lines / found in body fluids**

Source (cell line/ body fluid)	**EV Subtype**	**Features**	**Comments**	**Refs**
Human colorectal cancer model (LIM1863)	$A33^+$ exosomes	$A33^+$, $CD63^-$, $Alix^+$, $TSG101^+$	$A33^+/A33^-$ exosome subtypes were isolated from cell culture medium of human CRC LIM1863 cells using sequential immunoaffinity capture ($A33^-$ mAB / EpCAM-mAb loaded magnetic beads). Proteome profiling shows distinct protein signatures for $A33^{+/-}$ exosomes; $A33^+$ exosomes are selectively enriched in intracellular apical trafficking proteins and $A33^-$ exosomes, basolateral trafficking proteins. miRNA profiling[93] and mRNA transcriptome profiling[94] reveal distinct signatures. Moreover, sMVs were isolated (ultracentrifugation, 10,000g) and proteome analysis rvealed distinct profile to clearly distinguish A33- and EpCAM-exosomes: 462 proteins (33%) were found to be unique to sMVs. sMVs were enriched for members of the ATP-binding cassette superfamily (e.g. ABC transport proteins ABCB1, ABCB4, ABCC1, ABCC2, ABCE1, and ABCG2) that are typically found in microsomal and plasma membrane preparations.	[92]
	A33- exosomes	$A33^-$, $CD63^+$, $Alix^+$, $Tsg101^+$		
	sMVs (distinct from both exosome subtypes)	$KIF23^+$, $FLOT1^+$, $MYO1D^+$, $RACGAP1^+$		
Human melanoma (B16F10), squamous	Low-density	Both HD- and LD-exosomes display Alix, TSG101, CD9, CD81	HD- and LD-exosomes from a number of cell lines were isolated using density gradient (sucrose) centrifugation. HD-	[95]

carcinoma (A431), mouse heart endothelial (H5V), mesenchymal stem cell (immortalized human MSC hTERT), mouse neuroblastoma cells (N2a), human plasma	(LD) exosomes	and CD63 on their surface; buoyant density range of both subtypes 1.12-1.19 g/mL	and LD-exosome subtypes have unique protein and RNA compositions and have different functional effects on recipient cells.	
	High-density (HD) exosomes			
Human plasma (platelets)	Exosomes	40 – 100 nm (immuno-electron microscopy), CD63+	Platelet activiation resulted in formation of exosome and sMV release from ILVs and plasma membrane, respectively. immuno-electron microscopy of platelet aggregates revealed CD63+ internal vesicles in fusion profiles of MVBs, and in the extracellular space between platelet extensions. Functionally, these EVs were shown to be distinct, where annexin-V binding was restricted to sMVs; binding of factor X and prothrombin observed to the sMVs but not to exosomes.	[8]
	sMVs	100 nm - 1 μm (flow cytometry), integrin chains αIIb-β3 and β1, GPIbα, and P-selectin		
Mesenchymal stem cells (immortalized E1-MYC ESC-derived)	Cholera toxin B-chain (CTB)+ exosomes	CD81+, CD9+, Alix+, Tsg101+, fibronectin-, actin+	MSC-derived exosomes were isolated based on their respective affinities for the membrane-lipid binding moieties cholera toxin B chain (CTB), annexin V (AV) and Shiga toxin B subunit (ST) respectively. Proteome and RNA cargo of the 3 subtypes are distinctive	[308]
	Annexin V (AV)+ exosomes	CD81-, CD9-, Alix-, Tsg101-, fibronectin-, actin+		

	Shiga toxin B subunit+ exosomes	CD81-, CD9-, Alix-, Tsg101-, fibronectin+, actin+		
Human monocyte-derived dendritic cells	High density (HD) - exosomes	Buoyant density 1.15 g/mL,~50–<200 nm, MHCI+, CD9+ (WB), extracellular matrix proteins COL6A3, PEDF, SERPINF1 (proteomics)	Density gradient (iodixanol) centrifugation used to separate HD- and LD-exosome (100,000g pellet, small EV) and microvesicle (10,000g pellet, large EV) subtypes and define distinct protein signatures of each subtype. Proteins to be used potentially as markers of different EV subtypes, and further studies investigating the specific molecular machineries required for their biogenesis and secretion.	[104, 253]
	Low-density (LD)-exosomes	Buoyant density 1.11 g/mL, ~50–150 nm, MHCI+, CD9+, CD63+ (WB), plasma membrane, endosomal proteins, TSG101, SYN1, EHD4, ANNXI, ADAM10 (proteomics)		
	High density (HD) - sMVs	Buoyant density 1.17 g/mL,~150–<200 nm, MHCI+, CD9+ (WB), ribosome, mitochondrial and ER proteins, actinin-4, mitofilin (proteomics)		
	Low-density (LD)-sMVs	Buoyant density 1.11 g/mL,~150–<200 nm, MHCI+, CD9+, CD63+ (WB), ribosome proteins, SYN1,		

		EHD4, TSG101, ADAM10 (proteomics)		
Human saliva	Exosome I	Alix+, Tsg101+, CD63+, Hsp70+, CD26-	Human saliva-derived exosome-I and –II were fractionated using gel-filtration on Sephacryl S-500 and shown to have different size and protein composition. Most of CD26 (dipeptidyl peptidase IV (CD26) present in whole saliva is found in exosome II subtype and shown to be metabolically active in cleaving chemokines CXCL11 CXCL12.	[309]
	Exosome II	Alix+, Tsg101+, CD63+, Hsp70+, CD26+		
Human seminal fluid	Large diameter (105 +/- 25 nm) - exosomes	Buoyant density ~1.15 g/ mL ; CD9+, Psc+ , annexin A1+	Two distinct exosome (prostasomes) subtypes were isolated from seminal fluid using density (sucrose) gradient ultracentrifugation.	[310]
	Small diameter (56 +/- 13 nm) - exosomes	Buoyant density ~1.25 g/ mL;CD9+, Psca+, Glipr2+		
Rat basophilic leukemia-2H3 (RBL-2H3) cells	Exosome I	CD63+	Three distinct exosome subtypes identified by combining protein sorting (CD63, CD81, and MHC II) and different fluorescent lipid (phosphocholine, ceramides) probes that	[140]
	Exosome II	MHCII+		

	Exosome III	CD81+	label distinct cell compartments such as PM outer leaflet and the Golgi apparatus.	

Human metastatic melanoma (B16F10), human metastatic breast adenocarcinoma (MDA-MB-231) and human metastatic pancreas (AsPC-1)	exosomes -S	60–80 nm, Tsg101+, Alix+, Flot1+, Tollip+,	Use of AF4 (flow field-flow fractionation) to identify two exosome subpopulations and exomere nanoparticle type. Biophysical, proteomic, lipidomic, genomic, and glycomic (N-glycan) characterisation revealed selective differences in cargo of distinct EV subsets	[89]
	exosomes -L	90–120 nm, Tsg101+, Alix+, Vps4+		
	Exomere	~35 nm, Hsp90+, Mat1a+, Idh1+, Gmppb+		

Human colon cancer (LIM1863) cells	Exosomes (filtration <0.1 µm)	Exos: PDCD6IP/Alix, TSG101, CD81, CD63	Using an ultrafiltration approach to separate and isolate distinct EV subtypes (exosomes and sMVs) from culture medium, extensive biochemical and functional characterisation of these EVs was performed, demonstrating one subtype (fraction Fn1) comprised heterogeneous EVs with particle diameters of 30-1300nm, the other (fraction Fn5) being homogeneous EVs of 30-100nm diameter. Proteomics identified select and common marker proteins between the distinct EV subtypes. First report of 350 proteins uniquely identified in sMVs, many have the potential to enable discrimination of this EV subtype from exosomes (notably, KIF23, CSE1L, and RACGAP1). Both EVs shown	[67]
	sMVs (filtration >0.65 µm)	sMVs: KIF23, CSE1L, RACGAP1		

			to be induce invasion of recipient fibroblast cells, with sMVs shown to promote invasion significantly greater than exosomes	

Human normal bronchial epithelial cells and non-small cell lung cancer (NSCLC) cells	Exosomes	1.14–1.19 g/mL, 40–130 nm, Alix+, CD63+, TSG101+	Tandem-Mass-Tag (TMT) quantitative proteomics approach and Support vector machine (SVM) were employed to identified 251 proteins as "true" exosomal cargo proteins comparing with sMVs, crude exosomes and density-based (OptiPrep) exosomes.	[270]
	sMVs	10,000g fraction, PARP1+, CYC1+		

Human metastatic prostate cancer (DU145) cells	Exosomes	1.10 g/ml, 50-100 nm, CD9+, CD81+, TSG101+	SILAC proteomics identified differentially expressed and unique proteins in large oncosomes and exosomes, CK18 is highly expressed in large EVs compared with exosomes in this SILAC proteomic data. It led to demonstrated CK18 is a marker of large oncosomes in vivo.	[311]
	Large oncosomes	1.10-1.15 g/ml, >1 µm, CK18+, GAPDH and HSPA5, CD9-, CD81-, TSG101-		

Rat insulinoma cells (NHI 6F Tu28)	Exosomes	40–100 nm, CD81, CD82, CD63, TSG101, Alix	SILAC proteomics identified a large number of beta-cell specific proteins and new proteins from microvesicles generated cytokine-induced apoptosis. Differenbtial ultracentrifugation was used to selective isolate each EV subtype. Several cell death and cell signalling molecules was mapped using pathway analysis software.	[103]
	sMVs	200-600 nm, CD59, FLOT1, SNAP23, SNAP25		

Human blood, peripheral blood mononuclear cells (PBMCs)	Exosomes	Small EVs defined <200 nm, HSP70+, TSG101+, microtubule-associated proteins, and ubiquitinated proteins	EV subtypes were analysed from human lymphocytes following release stimuli (activation Vs. apoptosis induction). Proteomics identified select and common marker proteins between the distinct EV subtypes from T-Lymphocytes.	[312]
	sMVs	Large EVs defined 200-1000 nm, HSP70+, TSG101+, GSN, ESP+, EHD3+		

Table 2 - Overview of commonly used EV isolation methods

Method	Principle of separation	Purity	Integrity	Disadvantages	Advantages
Differential ultracentrifugation (DC)	Sedimentation velocity (size, volume, density) Typically used to isolate crude EVs mixtures Stepwise DC approach includes: initial 500g/2000g centrifugation (remove cells, membrane debris, apoptotic bodies), membrane filtration including 0.1 µm [37] or 0.22 µm membrane filtration [313], 10-14,000g to isolate crude sMVs [65, 66, 92], 100,000*g* to isolate crude exosomes.	Medium	High centrifugation shear forces may affect EV integrity/functionality	- time consuming - high heterogeneity/ low purity - co-purification with non-EV components expensive laboratory equipment - yield dependent on sample viscosity and concentration [314]	- low/medium recovery yield - most common applied methodology in field
Density-gradient centrifugation (DGC)	Buoyant density (density, size) Typically used for purification of EV populations. Use of discontinuous gradient of a solution of sucrose (or less-viscous iodoxinal, OptiPrep) [15, 263, 315]. Iodoxinal gradients are readily measured by refractive index [316] less toxic than sucrose in downstream functional cell assays [317], forms iso-osmotic solutions at all densities (preserves vesicle size) [318],and allow non-vesicular components to	High	Soft/mild force	- long procedure (~18 hours). - effect of the gradient forming molecules on the EV functionality is unknown - aggregates of large proteins and/or proteins that were non-specifically	- high purity - medium yield of EVs (sample loss during fractionation) - potential for EV subtype isolation - previously used in clinical settings [322, 323]

	be differentially fractionated [319]. Typically, use of DC (ultracentrifugation) at 100,000*g* to establish gradients. Different variations of DGC include float-down and cushion fractionation [313]. Use to separate subpopulations of EVs, including exosomes of low (1.12-1.19 g/ml) and high density (1.26-1.29 g/ml) [95, 104], and sMVs of low (1.09 g/ml) and high density (1.12 g/ml) [104].			associated with EVs are also being sedimented [320] - co-isolation of high-density lipoproteins (HDLs) [321] - low scalability	
Affinity isolation	Surface marker selectivity. The tag may be biospecific surface protein, such as a monoclonal antibody (mAb), that targets an EV-surface antigen, biospecific peptide (e.g., designer synthetic peptides with high affinity for HSPs [324], or proteoglycan affinity reagents (e.g., heparin [256, 304, 325]). mAbs that have been successfully employed as bait include those directed against A33 [262], EpCAM [37, 326], MHC-II antigens [327, 328], CD45 [329, 330], CD63 [331, 332], CD81 [332], CD9/CD1b/CD1a/CD14 [333], and HER2 [334]. Heparin affinity-based affinity capture [325] is generally applicable for EV isolation from cell culture media and biofluids, given it overcomes	High	Soft/mild force	- expensive (if antibody based). - EV elution might damage surface proteins and functionality. - typically dependent on availability of suitable mAbs directed to specific EV-surface antigens. - low scalability - low yield (binding capacity)	- high purity - potential to purify - different EV (sub)populations. - ability of coupling with other methods of characterisation (i.e., flow cytometry, western blotting and rt-PCR

	limitations with availability of suitable mAbs directed to specific EV-surface antigens.				
Size exclusion and gel permeation chromatography	Size, molecular weight) Approach has been widely applied for isolating EVs from plasma samples [335] and adapted (using commercially-available columns) for high-throughput clinical samples [336]. Gel permeation chromatography overcomes many of the problems associated with EV isolation from plasma/serum using DC/DGC – e.g., co-isolation of EVs with large-M_r protein aggregates and lipoproteins [335-337].	Medium/ high	Mild force	- concentration - dilution in elution buffer	- high scalability
Precipitation	Salting out using a polyethylene glycol/salt solution Approaches provides rapid, very impure EV preparations, unsuitable for detailed biophysical/ functional assay purposes. However, the method affords an isolation/concentration step for crude EV preparation for the purpose of diagnostic assay of known EV-associated biomarkers. Recent developments using sequential polyethylene glycol precipitation and adsorption to immobilized lectin	Low	Mild force	- low purity - PEG chain might envelope the EVs, possibly interfering with their functionality	- applicable for large volumes - experience from the viral field - recent advances in sequential precipitation/absorption have indicated potential for select types of

	concanavalin A [189] have demonstrated both exosomes and sMVs can be selectively enriched				EVs to be differentially isolated - previously used in a clinical setting (as an EV concentration step prior to UC) [338]
Sequential filtration	Membrane filtration (size, molecular weight) Nanomembrane ultrafiltration spin devices, equipped with low protein binding membranes (e.g., polyether sulfone or hydrophilic polyvinylidene difluoride, PVDF)), have been recently applied in clinical laboratories for the isolation of EVs from multiple, low-volume, urinary [339, 340] and blood plasma samples [341]. In combination with DC and DGC, nanomembrane ultrafiltration has enabled fractionation of highly-purified EV subpopulations; sMVs and exosomes from the same cancer cell origin [67].	Medium/ high	Mild force	- interference with membrane filter (yield and selectively of separation)	- medium stability

Figure 1

Extracellular vesicles (EVs) can be generated by different intracellular origins, namely exosomes (endosomal-derived) and shed microvesicles (or microvesicles, microparticles, ectosomes, oncosomes) (PM-derived) (A). Exosomes originate by inward budding of the membrane-bound vacuole (early endosome) which undergoes several changes as it matures to form a late endosome, and fusion of internal multivesicular compartments (MVB) with the PM. Multiple machineries are involved in biogenesis of intraluminal vesicles of MVBs and of exosomes. Endosomal sorting complex required for transport (ESCRT) components, in addition to lipids, and tetraspanins involved in ESCRT-independent mechanisms have been described. Members of the Rab GTPase family (RAB7/11/27A/31/35) and other factors such as SYKT6 and VAMP7 have been shown to modulate exosome secretion. Formation of shed microvesicles involves PM organization and redistribution of select lipid microdomains, local disassembly of the cytoskeleton network, and contraction of the actin– myosin machinery. Although limited insight into the molecular mechanisms of formation and release of shed microvesicles, various members including ARF6, acid sphingomyelinase activity, and some ESCRT components (ESRCT-I), and RhoA/ROCK signalling. For targeting and docking of EVs preferentially to recipient cells, various select mechanisms exist. This specificity can be attributed to protein surface receptors and adhesion molecules (i.e., tetraspanins, integrins, proteoglycans, and lectins) that are enriched in EV subpopulations (B). Integrins, extracellular matrix proteins, lectins, proteoglycans, or glycolipids on EVs facilitate their interaction and docking with cells expressing appropriate receptors on their surface. In target cells, EVs can interact and be internalised by multiple pathways, including dynamin-, PI3-kinase-, and actin polymerization–dependent phagocytosis, micropinocytosis, clathrin-/ caveolae-dependent endocytosis, and lipid raft-mediated endocytosis, direct membrane fusion, or phagocytosis (C).

Figure 2 – Endosomal and trafficking machineries involved in exosome biogenesis

Multiple machineries are thought to be involved in exosome biogenesis of ILVs and MVBs. Members of the Rab GTPase family have been shown to modulate exosome secretion and are thought to act on different MVBs along ESCRT-dependent and -independent endocytic pathways. Both ESCRT-dependent and -independent exosome endosomal mechanisms have been shown. ESCRT components are involved in an ESCRT-dependent intracellular pathway that traffics MVBs and their ILV contents to fuse with the plasma membrane and released as exosomes (MVB III), while other ESCRT-independent pathways (lipid-dependent and tetraspanin-dependent) have been described (MVB I-II). Exosome formation has been shown to be regulated by ESCRT-II, ESCRT-III, syndecan and VPS4; ALIX-dependent exosomes contain syndecan, syntenin, and ALIX [118]. Inhibition of nSMase (enzymes that hydrolyse sphingomyelin to ceramide) have shown a decrease in exosome release [120]. CD63-dependent mechanism has been attributed for ILV/exosome formation, targeting the EBV-encoded LMP1 protein to ILVs and allowing its subsequent release in exosomes [188]. Further, CD9 or CD82 (not CD63) have been shown to induce exosome secretion, although generated through a ceramide-dependent mechanism [185], in addition to CD81 [124]. In the context of ILV formation different tetraspanins have been proposed, including CD9 (knockout mice secrete fewer exosomes compared with wild-type mice [185]), CD81 [48], Tspan8 (alter exosomal protein and mRNA content [186]), and CD63 [187], and CD63-mediated ILV sorting LMP1 [188] and PMEL [123]. Moreover, other proteins, such as NEDD4 (Nedd-family interacting protein 4) have been implicated in promoting exosome secretion and targeting cytosolic proteins into exosomes [307]. Other mechanisms of endosomal regulation include ubiquitin-dependent/independent pathways, which generate highly specialized MVB-like organelles, use the same endosomal subdomains enriched in the same machinery that controls sorting of ubiquitin-dependent cargo into MVB/ILV pathway and release as exosomes [184]. Importantly, it has recently been shown that both ESCRT-dependent and -independent ILV formation can operate within the same MVB [187], with select processes regulating competing machineries generating subpopulations of ILVs which differ based on their size distribution and mechanism of formation.