Shedding light on the cell biology of extracellular vesicles.

Guillaume van Niel, Gisela d'Angelo, Graca Raposo

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Guillaume van Niel[1], Gisela D'Angelo[2] and Graça Raposo[2]

1. France Center of Psychiatry and Neurosciences, INSERM U895, Paris 75014, France

2. Institut Curie, PSL Research University, CNRS UMR144, Structure and Membrane Compartments, Paris F-75005

Correspondence should be addressed to: G.R graca.raposo@curie.fr

Abstract :

Extracellular vesicles are a heterogeneous group of cell-derived membranous structures that comprises exosomes and microvesicles, which originate from the endosomal system or are shed from the plasma membrane, respectively. They are present in biological fluids and are involved in multiple physiological and pathological processes. Extracellular vesicles are now considered as an additional mechanism for intercellular communication allowing cells to exchange proteins, lipids and the genetic material. Knowledge of the cellular processes that govern extracellular vesicle biology is essential to shed light on physiological and pathological functions of these vesicles as well as on clinical applications involving their use and/or analysis. Yet, many unknowns still remain in this expanding field related to their origin, biogenesis, secretion, targeting and fate.

[H1] Introduction

Apart from the release of secretory vesicles by specialized cells, which carry, for example, hormones or neurotransmitters, all cells are capable of secreting different types of membrane vesicles, known as extracellular vesicles, and this process is conserved throughout evolution from bacteria to humans and plants[1] [2,3]. Secretion of extracellular vesicles has been initially described as means of eliminating obsolete compounds[4] from the cell. However, now we know that extracellular vesicles are more than waste carriers, and the main interest in the field is now focused on their capacity to exchange components between cells — varying from nucleic acids to lipids and proteins — and to act as signalling vehicles in normal cell homeostatic processes or as a consequence of pathological developments[5,6,7].

Even though one generic term — extracellular vesicles – is currently in use to refer to all these secreted membrane vesicles, they are in fact highly heterogeneous (Fig. 1), which has largely hampered characterization and manipulation of their properties and functions. Insights into the biogenesis of secreted vesicles was provided by transmission and immuno-electron microscopy, and by biochemical means [8-10] . Based on the current knowledge of their biogenesis, extracellular vesicles can be broadly divided into two main categories: exosomes and microvesicles (Fig 1a).

The term exosome (which should not be confused with the exosome complex, which is involved in RNA degradation[11]) was initially used to name vesicles of an unknown origin released from a variety of cultured cells and carrying 5'-nucleotidase activity[12]. Subsequently, the term exosomes was adopted to refer to membrane vesicles (30-100 nm in diameter) released by reticulocytes **[G]** during differentiation[4]. In essence, exosomes are intraluminal vesicles (ILVs) formed by the inward budding of endosomal membrane during maturation of multivesicular endosomes (MVEs) — which are intermediates within the endosomal system — and secreted upon fusion of MVEs with the cell surface[13,14] (Fig 1a-c). In the mid 1990's exosomes were reported to be secreted by B lymphocytes [15] and dendritic cells[16] with potential functions related to immune regulation, and considered for use as vehicles in anti-

tumoral immune responses. Exosome secretion is now largely extended to many different cell types and their implications in intercellular communication in normal and pathological states are now well documented[5].

Microvesicles, formerly called “platelet dust”, were described as subcellular material originating from platelets in normal plasma and serum[17]. Later, ectocytosis, a process allowing the release of plasma membrane vesicles, was described in stimulated neutrophils[18]. Although microvesicles were mainly studied for their role in blood coagulation[19,20], more recently they were reported to have a role in cell–cell communication in different cell types, including cancer cells[21] where they are generally called oncosomes. Microvesicles range in size from 50-1000 nm in diameter, but can be even larger (up to 10µm) in the case of oncosomes. They are generated by the outward budding and fission of the plasma membrane and the subsequent release of vesicles into the extracellular space [22] (Fig 1a-c).

There is now evidence that each cell type tunes extracellular vesicle biogenesis depending on its physiological state, and to release extracellular vesicles with particular lipid, protein and nucleic acid compositions[5] (Fig. 1d). Because most published reports of extracellular vesicles have focused on their potential functions rather their origins, it is still unclear which sub-species of vesicles is responsible for any given effect. The current available protocols to recover extracellular vesicles from cell culture supernatants or liquid biopsies result in a heterogeneous population of vesicles of unknown origin [23]. Moreover, the diversity of isolated extracellular vesicle populations is further expanded by the inclusion of additional structures into the pool of extracellular vesicles, such as the apoptotic bodies, migrasomes, which transport multivesicular cytoplasmic contents during cell migration[24] or arrestin domain-containing protein 1-mediated microvesicles (ARMMS)[25], which are largely uniform, ~50 nm in diameter, microvesicles that have been shown to bud directly from the plasma membrane in a manner resembling the budding of viruses and dependent on arrestin domain-containing protein 1 (ARRDC1) and on endosomal sorting complex required for transport (ESCRT) proteins (similarly to a sub-population of exosomes; see also below).

The overlapping range of size, similar morphology and variable composition challenge current attempts to devise a more precise nomenclature of extracellular vesicles[26 27]. Nevertheless, novel isolation and characterization methods are being developed to allow a more thorough description of respective functions of the different types of extracellular vesicles and to establish a suitable classification and terminology. Moreover, to validate respective roles of exosomes and microvesicles, efforts are being made to uncover mechanisms underlying the targeting of the different cargoes that these vesicles transport to the site of extracellular vesicle biogenesis, the generation and secretion of vesicles, and their fate in target cells. Here, we review current knowledge and delineate unknown aspects of the essential cellular processes that govern the biology of mammalian extracellular vesicles, including their potential physiological roles, as well as their relevance to disease and to clinical applications.

[H1] Biogenesis of extracellular vesicles

Exosomes and microvesicles have different modes of biogenesis (but both involve membrane trafficking processes): exosomes are generated within the endosomal system as ILVs and secreted during fusion of MVEs with the cell surface, whereas microvesicles originate by an outward budding at the plasma membrane[10]. This nomenclature is still questionable as extracellular vesicle biogenesis pathways may differ according to the producing cell type. For example, T cells generate primarily extracellular vesicles from the cell surface with characteristics of exosomes, likely exploiting at the plasma membrane molecular components and mechanisms that are usually associated with the endosomal biogenesis of ILVs[28]. This peculiar biogenesis of exosomes from the plasma membrane might be specific to T cells, which also use the endosomal machinery for HIV budding at the plasma membrane[29].

Even though generation of microvesicles and exosomes occurs at distinct sites within the cell, common intracellular mechanisms and sorting machineries **[G]** are involved in the biogenesis of both entities. In many cases these shared mechanisms hinder the possibility to distinguish among them[5]. Mechanistic details of extracellular vesicle biogenesis have just started to be uncovered as discussed below. First, cargoes scheduled for secretion within extracellular vesicles must be targeted to the site of production, either at the plasma membrane (for microvesicles) or at the limiting membrane of MVE (for exosomes). Second, cargoes are enriched in the forming vesicles by a stepwise mechanism of clustering and budding followed by fission and vesicle release (Fig 2).

[H3] Cargoes and their targeting to the site of extracellular vesicle generation.

The nature and abundance of extracellular vesicle cargoes[30] (Fig 1d) is cell type specific and is often influenced by the physiological or pathological state of the donor cell, the stimuli that modulate their production and release, and the molecular mechanisms that lead to their biogenesis[31]. Cargoes are the first regulators of extracellular vesicle formation. As reported for exosomes, an ectopic expression of a particular cargo such as, for example the expression of the major histocompatibility complex **[G]** (MHC) class II[32] promotes MVE formation with a consequent release of extracellular vesicles, likely by recruiting sorting machineries that will promote MVE and ILV generation[32,33].

Exosomal membrane cargoes reach endosomes from the Golgi apparatus or are internalized from the plasma membrane before being sorted to ILVs during endosome maturation[34] (Fig 1). Hence cargoes that are preferentially recycled to the plasma membrane are likely not enriched in exosomes unless their recycling is impaired, as is the case for the transferrin receptor in reticulocytes[35]. Therefore, impairment or depletion of regulators of endosomal recycling and retrograde transport from endosomes to the Golgi might generally affect the targeting of some cargoes to extracellular vesicles. In this context, syntenin **[G]** protein, by acting both in the recycling[36] and in the sorting of syndecan **[G]** in MVEs[37] for exosome biogenesis, appears as a

potential regulator of the crossroad between endocytic recycling and endosomal targeting of potential exosomal cargoes.

Modulation of endocytosis or recycling of cargoes to the plasma membrane would also impinge on their targeting at the site of microvesicle biogenesis. For example, the small GTPase ADP-ribosylation factor 6 (ARF6) was identified as a regulator of selective recruitment of proteins, including β1 integrin receptors, MHC class I molecules, membrane type 1-matrix metalloproteinase 1 (MT1-MMP) and the vesicular soluble N-ethylmaleimide-sensitive factor attachment protein receptor (v-SNARE) VAMP3 into tumour-derived microvesicles[38,39]. In addition to ARF6-regulated endosomal trafficking, VAMP3 mediates the trafficking and incorporation of MT1-MMP into tumor-derived microvesicles in a CD9-dependent manner. This suggests that VAMP3- and ARF6-positive recycling endosomes are a site of MT1-MMP recycling to the cell surface and trafficking to microvesicles. Such crosstalk between recycling and microvesicle biogenesis is also illustrated by studies reporting that the small GTPase Rab22a co-localizes with budding microvesicles and mediates packaging and loading of cargo proteins in hypoxic breast cancer cells[40].

[H3] Machineries involved in the biogenesis of exosomes.

Exosomes are generated as ILVs within the lumen of endosomes during their maturation into MVEs, a process that involves particular sorting machineries. These machineries first segregate cargoes on microdomains of the limiting membrane of MVEs with consequent inward budding and fission of small membrane vesicles containing sequestered cytosol (Fig 2).

The discovery of the ESCRT machinery as a driver of membrane shaping and scission was the first breakthrough into uncovering the mechanisms involved in the formation of MVEs and ILVs[41]. ESCRT acts in a stepwise manner where ESCRT-0 and ESCRT-I subunits cluster ubiquitinated transmembrane cargoes on microdomains of the limiting membrane of MVEs and recruit, via ESCRT-II, the ESCRT-III subcomplexes that perform budding and fission of

this microdomain (Fig 2). Accordingly, Hrs (ESCRT-0) appears to be required for exosome formation and/or secretion by dendritic cells[42].

The role of the ESCRT machinery in ILV biogenesis and the presence of some ESCRT subunits in exosomes opened an avenue to understand and modulate the formation of exosomes through manipulation of the ESCRT components. A medium-throughput RNA interference screen targeting [23] different components of ESCRT machinery and associated proteins ~~highlights~~ has revealed various roles for selected members of this family in exosomes generation. Their inactivation impacts either the efficiency of secretion or the composition of the secreted vesicles indicating that some ESCRT components could act selectively on MVE and ILV subpopulations fated for secretion as exosomes[43]. The canonical ESCRT pathway can be intersected by syntenin and ALIX (ALG-2 interacting protein X; an ESCRT accessory protein; also known as PDCD6-interacting protein), which bridge cargoes and the ESCRT-III subunit vacuolar protein sorting-associated protein 32 (VPS32; also known as CHMP4)[37].

Exosomes can be also formed in an ESCRT-independent manner, which was revealed by studies showing that MVEs, featuring ILVs loaded with CD63 are still formed upon depletion of components of the four ESCRT complexes[44]. The first ESCRT-independent mechanism of exosome biogenesis was shown to require generation of ceramide **[G]** by neutral type II sphingomyelinase that hydrolyzes sphingomyelin **[G]** to ceramide[45]. Ceramide may then allow generation of membrane subdomains[46], which impose spontaneous negative curvature on the membranes. Alternatively, ceramide could be metabolized to sphingosine 1-phosphate to activate G_i-protein coupled sphingosine 1-phosphate receptor that appears essential for cargo sorting into exosomal ILVs[47] (Fig 2). In addition, proteins of the tetraspanin family **[G]**, have been shown to regulate the ESCRT-independent endosomal sorting. One of these proteins is CD63, which is particularly enriched on the surface of exosomes and has been shown to be involved in endosomal sorting in melanocytes[48,49] , in cargo (apolipoprotein E) targeting to exosomes secreted by melanoma cells[50] and in the biogenesis of exosomes in fibroblasts from down syndrome

patients[51]. Tetraspanins CD81, CD82 and CD9 are also directly involved in the sorting of various cargo to exosomes[52,53]. Mechanistically, these proteins form clusters and dynamic membrane platforms with other testraspanins and with different transmembrane and cytosolic proteins[54] likely acting in the formation of the microdomains that will bud. Moreover, recent structural analysis of the tetraspanin CD81 revealed a cone-like structure with an intramembrane cavity that can accommodate cholesterol and that is likely shared by other tetraspanins. Clustering of several cone-shaped tetraspanins could then induce inward budding of the microdomain in which they are enriched[55] (Fig 2). But tetraspanins also regulate the intracellular routing of cargoes towards MVEs, such as integrins[56], which indicates that impairment of their function may affect different steps of exosome generation. Thus, it seems that both ESCRT-dependent and -independent mechanisms operate in exosome biogenesis, and their contribution may vary depending on the cargoes, which recruit them, and the cell type.

As mentioned above, sorting of transmembrane cargoes into extracellular vesicles is largely dependent on endosomal sorting machineries. However, additional mechanisms contribute to the targeting of selective soluble or membrane associated cargoes to exosomes. For example, the sequestration of cytosolic proteins into ILVs can results from co-sorting with other proteins, such as chaperones heat shock protein 70 (HSP70) and heat shock cognate 70 (HSC70), which are found in exosomes derived from most cell types[57,58]. Membrane cargoes, such as GlycosylPhosphatidyInostol (GPI)-anchored proteins **[G]** are present in exosomes likely because of their affinity for lipid domains and lipid rafts **[G]** that could be directly involved in ILV generation through their effects on biophysical properties of membranes[59]. It has also been proposed that some cytosolic proteins, modified by ubiquitylation[60] or farnesylation[61] are segregated in ILVs and in exosomes but the underlying mechanisms for their enrichment in these compartments are still lacking. Apart from proteins, extracellular vesicles also carry nucleic acids, including RNAs (mRNAs and non-coding RNAs including micro RNAs (miRNAs)[62,63] and DNA sequences[64,65]. Interestingly, miRNAs have been shown to be differentially sorted to exosomes, depending on their sequence (presence of

specific motifs)[66], which indicates that incorporation of nucleic acids into exosomes is regulated . However, the relative contributions of passive and active loading of RNAs into extracellular vesicles remain unclear[67]. The mechanisms involved in targeting nucleic acids to exosomes are so far elusive. Different machineries have been proposed to perform specific nucleic acid sorting, including the ESCRT-II subcomplex that could act as an RNA binding complex[68], the tetraspanin-enriched microdomains that could sequester ~~many~~ RNA-binding proteins in the membrane subdomains[69] or the miRNA-induced silencing complex (miRISC) and protein argounaute 2 (AGO2), which mediate RNA silencing processes[70]. New regulators of miRNA sorting into exosomes have also recently been described and include the KRAS–MEK signalling pathway **[G]** acting through AGO2 [71], Major Vault protein **[G]** [72] or Y-box protein 1 **[G]** [73].

In sum, exosome biogenesis is certainly complex and varies depending on the cargo and on the cell type and can be influenced by other signals and pathological stimuli that the cell can receive. The balance of these pathways leading to changes in the compositional repertoire of exosomes also changes over the course of the differentiation process as reported for reticulocytes[74], or during cell maturation as shown for dendritic cells[75]. Accordingly, most cells host subpopulations of MVEs distinguished by different lipid and protein compositions and morphology[52,76]. In this context, different sorting mechanisms can act on the same endosomal compartment[49] or different machineries can be used for targeting the same cargo (for example MHCII, which can be targeted to MVEs by both ESCRT-dependent and independent mechanisms)[52,77], or on different maturation products of the cargo (as is the case for melanocyte protein PMEL for which its luminal domain, which is generated by proteolysis, is sorted by ESCRT-independent mechanisms, whereas ESCRT-dependent mechanism is involved in targeting the transmembrane domain of PMEL)[49]. Therefore several mechanisms could concomitantly or sequentially act on forming MVEs, thereby allowing the sorting of diverse cargoes at different stages of maturation of the MVE[78]; alternatively or concomitantly, distinct subpopulations of MVEs may exist and may be targeted by different machineries[5,49] (Fig 3). Overall, this data support

a model, whereby the biogenesis of exosomes involves several distinct mechanisms for the preferential recruitment of cargoes likely generating heterogeneous populations of ILVs and exosomes within common or distinct subpopulations of MVE[5,6].Overall, as major regulators of the composition of exosomes, endosomal sorting machineries appear as main determinants of their functional properties. Therefore, agents or activities affecting early endosomal sorting machineries and their dynamics should be considered when investigating exosome generation and for their manipulation.

[H3] Machineries involved in the biogenesis of microvesicles.

Whereas blebbing from the plasma during apoptosis has long been known to produce microvesicles in the form of apoptotic bodies[79], the release of microvesicles from the plasma membrane of healthy cells and the mechanisms involved in this secretion have only started to emerge recently. This biogenesis requires several molecular rearrangements within the plasma membrane, including changes in lipid components and protein composition, and in Ca^{2+} levels[31]. Ca^{2+}-dependent enzymatic machineries including aminophospholipid translocases **[G]** (flippases and floppases), scramblases **[G]** and calpain **[G]** drive rearrangements in the asymmetry of membrane phospholipids (exposition of phosphatidylserine from the inner leaflet to the cell surface), which causes physical bending of the membrane and restructuring of the underlying actin cytoskeleton, which favour membrane budding and formation of microvesicles[21,80] (Fig 2). A genetic defect in the activity of the lipid scramblase suppresses the exposure of phosphatidylserine on blood platelets, and the production of procoagulant-containing microvesicles[80]. However, even when the membrane lipid asymmetry is maintained, microvesicle biogenesis might proceed[81,82]. These observations suggest that other lipids, and the domains they form, contribute to microvesicle biogenesis. One important lipid component is cholesterol, which is abundant in microvesicles and pharmacological depletion of which impairs their generation in activated neutrophils[83].

In addition to lipids, cytoskeletal elements and their regulators are certainly required for microvesicle biogenesis. The activity of Rho family of small GTPases **[G]** and of the Rho-associated protein kinase (ROCK), which are important regulators of actin dynamics, induce microvesicle biogenesis in different populations of tumor cells[84]. As another example, in the enterocyte brush border **[G]** , myosin 1a distributed along the microvillar tips exerts plus end-directed force on the apical membrane, leading to the formation and release of gut microvesicles[85].

The biogenesis of tumor-derived microvesicles (oncosomes) is also tightly associated with metabolic changes, the so-called Warburg effect **[G]** [86]. In breast cancer cells, elevated glutaminase activity is dependent on Rho GTPases[87], and inhibition of its activity blocks microvesicle biogenesis. This suggest that formation and loading of microvesicles is linked to their metabolic capability and to the Rho-GTPase signalling pathway, even beyond its role in actomyosin regulation.

As for cargo targeting to exosomes, lipids and other membrane associated cargoes are localized to sites of microvesicle budding through their affinity for lipid rafts or as is the case for oligomeric cytoplasmic proteins, by their anchoring to plasma membrane lipids[88,89] — two mechanisms that are strikingly analogous to the budding of HIV and other retroviruses. Cytosolic components fated for secretion into microvesicles require their binding to the inner leaflet of the plasma membrane. This association is dependent on their respective plasma membrane anchors (palmitoylation, prenylation, myristoylation) and the establishment of high-order complexes, that concentrates them to the small membrane domains from which forming microvesicles will bud[88,89]. It is still unclear how nucleic acids, which are generally found in microvesicles, are targeted to cell surface. One possible mechanism revealed from studies of cancer cells suggests the involvement of conserved zipcode RNA sequence motifs **[G]** in the 3' untranslated regions in mRNA targeting into microvesicles[90], but the details of this process remain to be discovered.

[H1] The release of extracellular vesicles

Once formed, microvesicles pinch off from the plasma membrane whereas exosome secretion requires the transport and apposition of MVEs to the plasma membrane to fuse with and release ILVs (as exosomes) into the extracellular milieu. The different intracellular events leading to their secretion are likely to impose a time difference between generation and release of both types of extracellular vesicles. Release of microvesicles would be likely faster as cargoes only need to remain at the plasma membrane to be targeted to microvesicles and their subsequent release would directly follow their generation and fission. On the contrary, release of exosomes requires additional steps to sort cargoes to MVEs, then to ILVs and extrasteps to target MVEs to the plasma membrane and to prime them for secretion. Such difference could be relevant from a functional point of view as it imposes additional regulatory "checkpoints" for the secretion of exosomes as compared to microvesicles. Whereas in some cases, such as embryonic development, cell differentiation and in general during maintenance of physiological homeostasis the release could be constitutive, this process may also be subjected to further modulation by the physiological state of the cell and the requirement for the supply of key structural components or other mechanisms that would act as triggers for secretion such as the generation of immunological synapse **[G]** [52,91]. As the release of microvesicles is likely the direct consequence of their generation and fission, in the next sections we focus on exosome release and only summarize the few studies on potential mechanisms that could be involved in microvesicle secretion.

[H3] Avoiding MVE degradation.

MVEs are primarily destined to fuse with lysosomes for degradation. However, mechanisms preventing their degradation and allowing MVE secretion exist, thereby enabling exosome secretion (Fig 3 and 4). The regulation of the balance between degradative and secretory capacity of MVEs remains largely unexplored, but the setting of this balance undoubtedly impacts on cell function. For example, lysosomal degradation defects that promote exosome secretion have been shown to enable efficient elimination of unwanted and/or defective proteins such as amyloids in the context of

neurodegenerative diseases[92,93]. The impairment of lysosomal activity by inhibiting the endosomal proton pump V-ATPase also leads to an increase of exosome release[94,95], and, for example, has been shown to trigger apical secretion of Hedgehog **[G]** -related peptides through a multivesicular compartment in *Caenorhabditis elegans*[96].

Some insights into how the balance between targeting MVEs for secretion and degradation is established have recently emerged. A first level of regulation of this balance is likely imposed by the sorting machineries at MVEs. While the different components of ESCRT machinery have various effect on exosomes secretion[23] and generally associated with degradative MVE, the syndecan–syntenin–ALIX pathway seems to be restricted so far to exosome secretion[37]. On the same line, MHCII is targeted to MVEs fated for lysosomal degradation through ubiquitination (likely recruiting ESCRT machinery) while ubiquitin- (and likely ESCRT-) independent mechanisms target MHC II to MVEs fated for secretion[52,77]. The mechanisms underlying this balance are still unclear but involve components of various sorting machineries such as ESCRT-I component tumour susceptibility gene 101 protein (TSG101), whose ISGylation **[G]** favours lysosomal degradation (and thereby impairment of exosome secretion)[94], or the tetraspanin 6[97], overexpression of which slows down lysosomal degradation likely by recruiting sorting machinery that involves the syntenin pathway. These findings are in accordance with the involvement of ESCRT-independent machineries in the generation of MVEs fated for exosome secretion but not for lysosome degradation[49,52,98].

A similar balance exists between exosome secretion and macroautophagy — the process that drives degradation of superfluous or damaged cellular components in the lysosome to maintain cellular homeostasis and that promotes energy conservation under stress. More specifically, the fusion of MVEs with autophagosome would promote their degradation and prevent exosome secretion[99] (Fig 4). In this context, it has been shown that the prion protein (PrP) can promote exosome secretion by inhibiting autophagosome formation and it does so by interacting with caveolin **[G]** and modulating its

inhibitory effect on autophagosome formation[100]. Of interest, chemical inhibition of autophagy increases the recovery of autophagosome-associated proteins in the isolated exosomal pellet but not of exosome-enriched proteins[101]. This suggests that the capacity of MVEs to secrete exosomes is counter-balanced by their fusion with the autophagosome. Yet, autophagosomes and MVEs can both secrete their content but the molecular mechanisms regulating these secretory pathways are likely distinct.

[H3] Transport of MVEs.

As discussed above, MVEs fuse either with lysosomes for degradation of their content or with the plasma membrane. In both cases a two-step process involving their transport (motility) and fusion is required, but the effectors involved in targeting MVEs to the lysosomes or to the plasma membrane are certainly distinct.

In general, intracellular transport involves the association of organelles with the cell cytoskeleton (actin, microtubules), associated molecular motors (dynein, kinesins, myosins) and molecular switches (Small GTPases)[102,103]. Exosome secretion is provided by the oriented secretion of these vesicles towards the immunological synapse between antigen-presenting cells and T cells during antigen presentation[52,104]. This implies that at least in the context of immunological synapse MVEs follow the network of microtubules oriented by the microtubule organizing centre (typically the centrosome)[91] (Fig 4). The molecular motors involved in this process remain to be determined but certainly counterbalance those that regulate transport of MVEs towards lysosomes. Targeting to lysosomes occurs by a retrograde transport on microtubules (towards microtubule minus ends), and Rab-GTPase Rab7 and its associated proteins promote the recruitment of the retrograde molecular motor dynein that targets MVE to lysosomes[105]. Interestingly, Rab7 is also mandatory for the release of exosomes[37]. These dual effect on exosome secretion seems to rely on the ubiquitylation status of Rab7, which has been shown to promote the recruitment of the machinery involved in lysosomal targeting of MVEs at the expense of exosome secretion[106]. Curiously, in endosomes the recruitment of Rab7 leading to lysosomal targeting is

stimulated by cholesterol at their limiting membrane, whereas MVE-containing ILVs enriched in cholesterol have been shown to undergo preferential secretion as exosomes[107]. Thus, dynamic changes in the composition of the limiting membrane of MVEs, through incorporation of specific lipids and proteins into ILVs, would likely regulate the fate of MVEs towards degradation or secretion.

Rab27a and Rab27b[32] and their respective effectors, synaptotagmin-like protein 4 and exophilin 5, are also essential for exosome secretion. Rab27b regulates the motility of MVEs towards the plasma membrane, and both Rab27 isoforms act on the step following MVE transport, that is the docking at the plasma membrane to promote fusion, thereby increasing exosome secretion**.** The role of Rab27a in MVE docking involves rearrangement of sub-membrane actin cytoskeleton[108], a step that is common to all mechanisms involving vesicular secretion. Rab27 also controls secretion of secretory lysosomes so called lysosome related organelles[109], which suggests that MVEs capable of exosome secretion may be considered as a specialized compartment rather than a simple MVE subtype. Of note, Rab27 isoforms are not constitutively expressed in all cell types, which implies that each cell type may adapt its own secretory machineries for exosome secretion. This is illustrated by reported involvement of additional Rabs and their effectors, such as Rab11 and Rab35 effector[110,111] in the direct regulation or the potential priming of MVE secretion.

[H3] Fusion of MVEs with the plasma membrane.

The final step of exosome secretion requires the fusion of MVEs with the plasma membrane to release ILVs as exosomes (Fig 4), a process likely mediated by SNARE proteins **[G]** and synaptotagmin family **[G]** members[112]. A SNARE complex known to be implicated in the exocytosis of conventional lysosomes consists of VAMP7 on the lysosomes, syntaxin 7 on the plasma membrane and the lysosomal regulatory protein synaptotagmin 7[113]. This complex is involved in exosome secretion in some cells (human leukemia K562 cell line)[114] but not in others (MDCK cells)[115]. The process of exosome secretion has been demonstrated in several cell types to be regulated by Ca^{2+}

[116-118], which may have a role in the activation of the SNARE complexes. The implication of SNAP23 — a SNARE shown to regulate lysosome-related organelles secretion in mastocytes[119] — also in exosome secretion[120], strengthens the notion that MVEs are indeed specialized secretory organelles. Additional SNARE proteins involved in exosome secretion such as YkT6 [121] in Drosophila, SYX-5 in *C. elegans* [122] and syntaxin 1a [123] in mammals reflect again the diversity of regulators that could be involved in exosome secretion, most likely depending on the organism, the cell type or the MVE subtypes. It should be noted that most of the studies on the intracellular regulators of exosome release came from analysis of exosomal pellets isolated from supernatants from cell cultures treated with inhibitors or interfering RNAs against potential targets, ignoring the complexity of intracellular pathways that might be affected in the producing cells by these perturbations. Moreover, the quantity of extracellular vesicles recovered in the supernatant does not take into account the fraction of vesicles that remains tethered (not fully released) at the plasma membrane of the producing cells[95] or the fraction of extracellular vesicles that can be recaptured by the same cell[124]. A better understanding of this step certainly requires the development of new tools and techniques to follow docking and fusion of MVEs with the plasma membrane.

[H3] Release of microvesicles.

The release of microvesicles requires their fission from the plasma membrane, a mechanism that is dependent on the interaction of actin and myosin with a subsequent ATP-dependent contraction[85,125]. As such, the activation of small GTP binding proteins including ARF6 and ARF1 leads to the phosphorylation of the myosin light chain (MLC) and actomyosin contraction, which allows the vesicles to bud off from the membranes of cancer cells[39 126 127]. In HeLa cells another regulator of actin dynamics, Cdc42 has been shown to be involved, but the underlying mechanism is still not known[84]. Interestingly, TSG101 and VPS4-ATPase, mostly involved in exosomes generation as part of the ESCRT machinery, were reported to participate in the scission and release of ARMMs (subtype of microvesicles containing ARRDC1)[25]. Shedding of ESCRT-dependent microvesicles was also reported in *C. elegans* embryos upon loss of the conserved flippase P4-

ATPase, TAT-5, which leads to the cytosolic exposure of phosphatidylethanolamine, an aminophospholipid asymmetrically enriched in the inner leaflet of the membrane bilayer[128]. This scenario mirrors the exposure of phosphatidylserine by lipid translocation, which as discussed above, can promote membrane bending and microvesicle budding. (Fig 2)

The involvement of cell signalling pathways in microvesicle release is strongly supported by reports showing that removal of serum, and therefore growth factors acting on their respective receptors and downstream effectors, prevents microvesicle release[129]. What is known is that a strong microvesicle release is induced by increased concentration of Ca^{2+}, which by activating scramblase and calpain leads to a loss of membrane phospholipid asymmetry and the reorganization of the cytoskeleton (see above) or by the activation of protein kinase C **[G]** by phorbol esters[130]. Release of microvesicles has also been shown to depend on ATP-mediated activation of $P2x_7$ receptors **[G]** , which leads to rearrangements of the cell membrane[131,132]. Mechanistically, this process is associated with the translocation of the acidic sphingomyelinase to the plasma membrane where it generates ceramide, thereby promoting membrane bending and microvesicle shedding[133]. The involvement of acidic rather than neutral sphingomyelinase in microvesicle release suggests that different members of the sphingomyelinase family control the biogenesis of exosomes[45] (see above) and the release of microvesicles, but in both cases, these mechanisms would support ESCRT - independent vesicle release.

[H1] Targeting to recipient cells

Once released into the extracellular space extracellular vesicles can reach recipient cells and deliver their content to elicit functional responses and promote phenotypical changes that will impact on their physiological or pathological status. Extracellular vesicle-mediated intercellular communication requires docking at the plasma membrane, followed by the activation of surface receptors and signalling, vesicle internalization (endocytosis) or their fusion with target cells (Fig 5). The mode of vesicle interaction with the cell surface and the mechanisms that mediate the transfer of extracellular vesicle

cargoes are not fully unravelled. These processes are complex and depend on the origin of extracellular vesicles and on the identity and origin of the recipient cells, as well as seem to be linked to the downstream effects and processes instigated by these vesicles[134]. Current studies have been mostly focused on investigating membrane interaction and intercellular fate of pools of exosomes, but despite different content and size, the principles of uptake and general intercellular trafficking of different sub-populations of extracellular vesicle are likely to be shared.

[H3] Binding of extracellular vesicles to their target cells.

Target cell specificity is likely to be determined by specific interactions between proteins enriched at the surface of extracellular vesicles and receptors at the plasma membrane of the recipient cells, as for example, in follicular dendritic cells **[G]** [135],intestinal epithelial cell[136], dendritic cells[137], or neurons [138], and also in liver, lungs or lymph nodes[136,139]. Of note, the recipient cells can also be the producing cell itself, leading to autocrine responses[124].

Several mediators **[Au:OK?]** OK of these interactions are known and include tetraspanins, integrins, lipids, lectin **[G]** or heparan sulfate proteoglycans **[G]**, and extracellular matrix (ECM) components (Fig 5 inset). The cellular and molecular basis for the specific targeting to acceptor cells is still unclear, although some data is available. For example, integrins on extracellular vesicles can interact with adhesion molecules such as intercellular adhesion molecules **[G]** (ICAMs)[140] at the surface of recipient cells. In addition, the interaction of integrins with extracellular matrix proteins, mostly fibronectin and laminin, has been shown to have important roles in exosome[141,142] and microvesicle [143] binding to recipient cells. In this context, the ECM can act as a "zipper" between integrins present on extracellular vesicles and target cells. *In vivo*, integrin heterodimers may drive extracellular vesicles towards specific target organs[139]. One example includes exosomes released by cancer cells, which can be targeted to specific organs such as lung and liver, to promote premetastatic niche formation in a manner dependent on their integrin

612 composition[139]. Exosomal tetraspanins could also regulate cell targeting. They
613 have been shown to interact with integrins[144] and to promote exosome
614 docking and uptake by selected recipient cells[145,146]. Other molecules such as
615 heparan sulphate proteoglycan and lectins, both present in extracellular
616 vesicles and at the plasma membrane, contribute to their docking and/or
617 attachment of these vesicles to recipient cells (Fig 5). Glypican-1, a cell
618 surface proteoglycan that bears heparan sulfate, and CD44, a cell-surface
619 glycoprotein involved in cell–cell interactions, are involved in exosome[147] and
620 microvesicle[148] docking, respectively. The lipid composition of extracellular
621 vesicles can also have an impact on recipient-cell targeting. For example,
622 phosphatidylserine can recruit specific lipid-binding proteins such as Galectin
623 5 or Annexin 5[140 149,150] that then induce docking of vesicles to the target cell
624 membrane.
625
626 ***[H3] Uptake and intracellular fate of extracellular vesicles.***
627 Once they have bound to recipient cells extracellular vesicles may remain at
628 the plasma membrane [135]; [52] or may be internalized by clathrin-mediated or
629 clathrin-independent endocytosis, such as macropinocytosis **[G]** and
630 phagocytosis[151-153] as well as through endocytosis via caveolae and lipid
631 rafts[157-159] (Fig 5). Of note, certain cell types, such as HeLa cells or EBV-
632 transformed B cell line release clusters of exosomes, as a result of tethering
633 the vesicles by protein tetherin[95]. This clustering may affect the way these
634 vesicles are internalized, favouring phagocytosis or macropinocytosis to
635 support the engulfment of such large masses or aggregates of extracellular
636 vesicles[151].
637
638 Specific composition of extracellular vesicles will influence their fate. The
639 presence of Amyloid precursor protein on one exosome subtype from
640 neuroblastoma cells will specifically target them to neurons contrary to a
641 CD63 enriched exosome subtype that binds both neurons and glial cells[154].
642 Another example is the presence of syncitin at the surface of exosomes
643 derived from the trophoblast **[G]** that promotes their uptake [155], whereas the
644 presence of a “don’t eat me” signal , such as CD47, at the surface has been

shown to have a strong inhibitory effect on vesicle phagocytosis by monocytes [156].

The fate of extracellular vesicles is also likely related to the presence of specific structures at the plasma membrane of the target cell. As an illustrative example, it has been shown that microvesicles derived from microglia **[G]** show largely different dynamics of interaction with membranes of microglia and astrocytes **[G]** [157]. It has also been shown that filopodia **[G]** drive extracellular vesicles toward sites of uptake[158]. The lipid composition of the plasma membrane of recipient cells such as the presence of lipid raft also contributes to extracellular vesicle internalization as disruption of lipid rafts by cholesterol depletion reduces uptake of extracellular vesicles[159].

Following interaction with the plasma membrane of recipients cells[157] and after uptake by different mechanisms, extracellular vesicles follow the endocytic pathway and reach MVEs, which in most cases, are targeted to the lysosome[160,161]. In some cases the internalized vesicles may escape digestion by back fusion with the limiting membrane of the MVE, thereby releasing their content into the cytoplasm of the recipient cell[162], a process that is still poorly understood but of prime importance to release intraluminal nucleic acid structures (Fig 5). The restricted co-localization of trophoblast-derived exosomes with early but not late endosomal structures also suggests that some internalized extracellular vesicles could escape lysosomal degradation by being re-secreted either via the early endocytic recycling pathway or by fusion of MVEs with the plasma membrane (Fig 5) [155].

Advances in live imaging methods and super-resolution techniques will surely aid in providing further understanding of the processes of extracellular vesicle uptake and their intracellular fates.

[H3] Signals delivered by extracellular vesicles to recipient cells.

Once docked at the plasma membrane, extracellular vesicles can elicit functional responses by binding to and activating receptors expressed on the recipient cells (Fig 5). First examples were B cells and dendritic cells derived

679 exosomes that were able to present antigen to T cells and induce specific
680 antigenic response[15,16]. Tumour derived microvesicles were shown to carry
681 fibronectin, which when bound to integrin on non-transformed fibroblasts was
682 able to promote their anchorage independent growth (one of the hallmarks of
683 tumorigenesis), contributing to the acquisition of transformed phenotype by
684 healthy cells[163]. As another example, microvesicles generated and released
685 by embryonic stem cells were shown to induce invasion of maternal tissue by
686 the trophoblast, which is mediated by the interaction of laminin and fibronectin
687 on the microvesicles with integrins along the surfaces of the trophoblast, and
688 which promotes embryo implantation[164]. The role of extracellular vesicles in
689 the long-range transfer of morphogens to recipient cells in developing
690 organisms was also shown[165].
691
692 Cargo delivered by extracellular vesicles can also activate various responses
693 and processes in the recipient cell after internalization. For example, in
694 dendritic cells, protein cargo of exosomes derived from intestinal epithelial
695 cells[136] or other dendritic cells[140] is processed in the endocytic compartment
696 similarly to antigens and then used in antigen presentation, thereby
697 contributing to immune response regulation. Extracellular vesicles could also
698 fuse directly with the plasma membrane or with the endocytic membrane of
699 recipients cells. Such processes are mandatory to release intraluminal content
700 in the cytoplasm of recipient cells, a key step to support the release of
701 miRNA[62] and mRNA[166] from extracellular vesicles into recipient cells to
702 regulate gene expression. Direct fusion of extracellular vesicles with the
703 membrane of recipients cells allow also the exchange of transmembrane
704 proteins and lipids. Extracellular vesicles can transport various lipid species
705 including eicosanoids, fatty acids, and cholesterol as well as lipid
706 translocases, thereby contributing to the regulation of bioactive lipid
707 species[167]. Under pathological situations, a good example of material
708 transferred through extracellular vesicles is given by pathological amyloid
709 proteins, which can be either enriched at the surface of extracellular vesicles
710 such as prion protein or amyloid beta peptide, or present intraluminaly such
711 as TDP43 and alpha-synuclein. Their transfer to recipient cells, requiring back
712 fusion, has been proposed to favour transcellular spreading of amyloids[168].

Mechanisms governing fusion of extracellular vesicles with these different compartments are not known yet, but could be analogous to fusogenic processes employed by viruses[169].

The ultimate and likely most frequent fate of extracellular vesicles is their targeting to lysosomes, which leads to the degradation of proteins and lipids carried by extracellular vesicles. Of importance this degradative pathway would provide a relevant source of metabolites to the recipient cells[170] (Fig 5).

[H1] Conclusions and perspectives

Much progress has been made in recent years in understanding the basic biology of extracellular vesicles, but further investigations are required to fully resolve the functional capabilities of these vesicles. Extracellular vesicles are involved in several physiological contexts and pathological states, including blood coagulation, inflammation, stem cell expansion, neuronal communication and tumorigenesis among others[6]. In this context, extracellular vesicles have been shown to carry, for example, tumour-associated molecules in case of cancer and premetastatic niche establishment[139 171], or particular components associated with neudegenerative diseases[172]. Thus, extracellular vesicles hold a great potential for clinical application.

Regulatory pathways involved in biogenesis and secretion of extracellular vesicles, when well defined, could be used to manipulate extracellular vesicle generation in pathological states, such as tumorigenesis, where the involvement of extracellular vesicles in pathology has been particularly well documented[163]. Nevertheless, it should be noted that manipulation of machineries involved in the biogenesis, transport or targeting of extracellular vesicles for therapeutic benefit should be approached with caution, owing to potential secondary effects of such manipulations on healthy tissues[173].

The broad and increasing interest in extracellular vesicles has also opened up the possibility to use exosomes and microvesicles as biomarkers to follow progression of various pathological states, for example for assessing risk of tumour progression and metastasis or for providing early biomarker of

neurodegenerative diseases[172]. Investigations in this area have flourished, aiming to put on solid ground the use of extracellular vesicles as biomarkers in a variety of diseases. Developing techniques to enrich for disease-associated (for example, tumour-derived) extracellular vesicles to define their selective cargo can improve the sensitivity of such biomarkers[174]. Whether these “membrane biomarkers” correspond to endosomal-derived exosomes or membrane-derived microvesicles is so far unclear although potentially informative. Future studies and optimized isolation procedures (Box 1) will shed light on the nature of the different extracellular vesicle subpopulations that could be associated with distinct pathological states and stages of progression of a given disease.

Another emerging application is the use of microvesicles and exosomes as vectors for the delivery of defined compounds or more generally for modulation of cell functions in an *in vivo* context. Extracellular vesicles are biocompatible, can be immunologically inert, and can, if necessary, be patient-derived and therefore with lower propensity to trigger innate and adaptive immune responses [175]. Their use in clinical research have already demonstrated that extracellular vesicles secreted by immune cells (dendritic cells) stimulate the immune system and can therefore be exploited as anti-tumor vaccines[176,177]. Several clinical trials involving the use of extracellular vesicle-based delivery are ongoing, for example for the treatment of lung cancer and melanoma, that may become part of an immunotherapy approach that has great potential for patients with advanced cancers[178]. Given that extracellular vesicles (in particular exosomes) can be either immunostimulatory or tolerogenic (immunologically inert), there are several examples of possible therapeutical interventions where extracellular vesicles can be used (reviewed in detail elsewhere[5,179],[172]). Beside the aforementioned use of extracellular vesicles in antitumoral therapy, dendritic cells pulsed with *Toxoplasma gondii* release extracellular vesicles that confer protection against subsequent toxoplasma infection[180]. Such strategy could be considered for fungi, bacteria, parasitic protozoa and helminths[172]. Mesenchymal stem cells-dreived extracellular vesicles are now tested in animal models to treat acute kidney failure[181], myocardial infarction[182] or

ischemia[183]. Other undergoing assays are based on *in vitro* manipulation of extracellular vesicles with the loading of a particular cargo (for example interfering RNAs; suicide mRNA/protein **[G]** , miRNAs, drugs) to then deliver it to the target cell as a drug or for bioengineering purposes[184,185]. Modulating the specificity of targeting extracellular vesicles to recipient cells will be key for their use as high precision vehicles, and such approaches have already been tested to optimize the delivery of siRNAs to the brain[184].

Despite the enormous therapeutical potential, the field is still in demand of new *in vivo* models combined with powerful imaging methods to track at the single vesicle scale, the release, trafficking routes and fates of extracellular vesicles within the complex architecture of the organism (see also Box 1). Cell biologists and physicians working side by side in a complementary manner will certainly shed further light on the basic functions of extracellular vesicles and on their translation from the bench to bedside.

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Display items

Box 1

Methods of isolation and analysis of extracellular vesicles.

The release of extracellular vesicles in the extracellular space allows their recovery from cell culture supernatants and liquid biopsies. Isolation procedures include differential ultracentrifugation, flotation on density gradient, separation by size exclusion chromatography, poly(ethylene glycol) (PEG) precipitation, immunoprecipitation and commercial kits that are partly based on these methods. These steps allow to concentrate and to separate extracellular vesicles from protein aggregates, lipoparticles, viruses, and cell debris with different rates of success. Combination of different isolation procedures is encouraged to notably separate subpopulations of vesicles based on their size, density or composition.

Several analytical methods are available and should be combined to first assess purity, integrity and concentration of extracellular vesicles before further analysis or other experiments. The most commonly used approaches for the analysis of the composition and morphology of extracellular vesicle populations include western-blot analysis, nano-particle tracking, transmission electron microscopy and flow cytometry and can be completed by proteomics, lipidomics and RNA and/or DNA sequencing[67,186]**.** Functional analysis of extracellular vesicles depends on the question to be addressed. It should always be performed after assessment of the purity of the extracellular vesicle pellet, as soluble proteins such as cytokines, protein complexes and aggregates or lipoparticles are a major source of false positive results in functional assays.

A crowdsourcing knowledgebase (http://evtrack.org) that centralizes extracellular vesicle studies and methodologies provide a means to standardize extracellular vesicle research to strengthen reproducibility between studies[187]. Emerging strategies are now developed to investigate biogenesis, uptake of extracellular vesicles and the transfer of material to recipient cells *in vitro* and *in vivo*. These strategies are mainly based on the

labelling of isolated extracellular vesicles with fluorescent dyes or expression of fluorescent reporters that are targeted to these vesicles. Such labelling has some limitations[188] but allows the tracking of extracellular vesicle by live cell imaging in cell lines and, to limited extent, also *in vivo*[189]. The main limitation being the size of the vesicles, super-resolution microscopy is one option to assess vesicle budding at the plasma membrane or in multivesicular endosomes, and to track their fate in recipient cells. A second approach is based on the loading of extracellular vesicles with molecules (mRNA, micro RNA, Cre recombinase)[190,191] which induce detectable signals such as modulation of expression of a reporter gene once released in recipient cells. Alternative approach using optical tweezers allows the manipulation and the visualization of single vesicle at the surface of recipient cells[157]. But, so far, the field is still in demand of *in vivo* models that would allow reproducible tracking of extracellular vesicles at a single vesicle and high spatio-temporal resolution at different stages: through their biogenesis and transit routes in secreting cells to their delivery and fate in the recipient cells. Such approach would overcome the actual limitations linked to, for example, the biased recovery of extracellular vesicles from the supernatant (resulting from processes such as vesicle tethering to the membrane of the producing cell or their re-uptake)[95,124]. But as in any novel field of research, pre-analytical and analytical methods for studying extracellular vesicles are bound to evolve and to be better standardized to render the increasing numbers of publications in this field comparable.

Figure Legends

Figure 1: Main features of extracellular vesicles.

(a) Extracellular vesicles comprise a heterogeneous population of membrane vesicles of various origins. Their size may vary (typically between 50 to 500 nm but they can be even larger measuring 1-10 um). Through the last two decades, extracellular vesicle have been denominated according to their origin (cell type), size, morphology and cargo content but can now be classified into two distinct classes: exosomes and microvesicles. (b) Extracellular vesicles are formed either by budding of the plasma membrane,

and are referred to as microvesicles or as intraluminal vesicles (ILVs) within the lumen of multivesicular endosomes (MVEs). MVEs fuse with the plasma membrane to release ILVs that are then called exosomes. (c) Processing of extracellular vesicles for observation by conventional transmission electron microscopy (TEM) causes their shrinking leading to an artefactual cup-shaped morphology (top panel). But when observed in close to native state by cryo electron microscopy (cryo-EM) they appear as round shaped double-leaflet-membrane enclosed structures (bottom panel). (d) Study of extracellular vesicle composition revealed that they can carry versatile cargoes, including proteins, lipid and nucleic acids and this content can largely vary between cells and conditions. The particular composition will directly affect the fate and function of extracellular vesicles, strengthening the importance of selective cargo sorting mechanisms. Of note, according to the cell type extracellular vesicles will display a set of cell type specific proteins that account for their specific fates and functions. Despite a different mode of biogenesis, exosomes and microvesicles display similar appearance, overlapping size and often common composition that make it difficult to ascertain their origin once isolated from the extracellular medium or from biological fluids. ARMMS, arrestin domain-containing protein 1-mediated microvesicles; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; ICAM, intercellular adhesion molecule; TSPAN: tetraspanin; LBPA: lyso-bis-phosphatidyl acid, ICAM: InterCellular Adhesion Molecule, MHC major histocompatibility Complex, APP: amyloid precursor protein, PMEL: premelanosomal protein, TCR: T cell receptor, HSPG heparan sulfate proteoglycan, CXCR4: C-X-C chemokine receptor type 4, PrP, Prion Protein, TfR: transferrin receptor, ALIX: ALG-2 interacting protein X, Tsg101 Tumor suppressing gene 101, vps: vacuolar protein sorting, LFA-1: Lymphocyte function associated antigen 1., TDP43: TAR binding protein 43, GAPDH: glyceraldehyde-3-phosphate dehydrogenase, ERK: Extracellular signal-regulated kinases, PLD: Phospholipase D. Images in part c are the courtesy of Roberta Palmulli (G. Raposo' laboratory, URM144, Institut Curie) for conventional TEM and of Daniel Levy (UMR168, Institut Curie).

Figure 2: Biogenesis of extracellular vesicles

Several sorting machineries are involved in the different steps required for exosomes and microvesicles generation. First, lipids and membrane-associated proteins are clustered in discrete membrane microdomains of the plasma membrane for microvesicles (top) and the limiting membrane of the multivesicular endosome (MVE) for exosomes (bottom) **(step 1).** Such microdomains certainly also participate in the recruitment of soluble components fated for sorting in extracellular vesicles such as cytosolic proteins and RNA species **(step 2)**. Altogether formation of these clustered microdomains together with additional machineries promote membrane budding followed by a fission process either at the plasma membrane towards the extracellular medium or at the limiting membrane of the MVE towards the lumen of MVE **(step 3)**. Transmembrane proteins sorted on exosomes and microvesicles keep the same topology as at the plasma membrane. Mechanisms of exosome biogenesis are relatively well understood and importantly involve subunits of endosomal sorting complex required for transport (ESCRT), although to a different degree — ESCRT-III is required for the scission of the intraluminal vesicles (ILVs) into the MVE lumen, but membrane budding can occur by either ESCRT-dependent or ESCRT-independent mechanisms. The mechanisms involved in the biogenesis of microvesicles is still expanding. Notably, the molecular machineries that act at the different steps of extracellular vesicle biogenesis are at least partly common to exosomes and microvesicles (prominently including ESCRT proteins but also generation of ceramide through the action of different types of sphingomyelinase). One exception is the flipping of specific lipid species between the leaflets of the budding membrane that has been uniquely reported so far to microvesicle budding. Thus, it is difficult to ascertain the origin of the produced extracellular vesicle by simply impairing the function of a given mechanism involved in biogenesis of these vesicles. ARF6, ADP-ribosylation factor 6.

Figure 3: Origin of exosome diversity in relation to sorting machineries.

The diversity of extracellular vesicle sub-populations with distinct composition and function that are generated by a given cell type is often attributed to the

production of distinct sets of exosomes and microvesicles[23,192]. This diversity is well-exemplified by the secretion of different exosomes sub-populations — with distinct morphology and composition — from apical and basolateral sides of polarized cells such as intestinal epithelial cells[193 194 195]. Such exosome sub-populations are likely originating from sub-populations of MVEs. The distinct composition of these subpopulations reflects on the presence of multiple sorting machineries that act on the MVE compartment. It remains to be determined whether the different sorting machineries act on distinct sub-populations of MVEs (see part on right) or concomitantly in single MVEs to generate distinct sub-populations of intraluminal vesicles (ILVs) (see part on the left). The reality is most likely a mix of these two possibilities, with distinct contribution depending on the cell type. Recruitment of a given sorting machinery can depend on the maturation stage of the producing cells[52 74], on the post-translational modification of the cargo (for example: proteolytic processing, ubiquitylation) [49,193] or potentially on the stage of maturation of MVEs. The type of sorting machinery recruited to MVEs will also specify the fate of MVEs between exosome secretion and lysosomal degradation. ESCRT, endosomal sorting complex required for transport.

Figure 4: Interdependency of intracellular trafficking routes in the generation of extracellular vesicles

The generation of exosomes and microvesicles requires a tuned regulation of multiple intracellular trafficking steps (blue arrows for exosomes~~,~~ green arrows for microvesicles) that influence the targeting of cargoes to the site of extracellular vesicle biogenesis as well as for exosomes, the fate of the multivesicular endosome (MVE) from which these vesicles originate. Cargoes targeted to MVEs originate from endocytosis at the plasma membrane or are directly targeted to MVEs or to early sorting endosomes via the biosynthetic pathway (from the trans-Golgi Network (TGN)). Retrograde transport towards the TGN or recycling back to the plasma membrane will divert cargoes from their targeting to MVE (dashed arrows) and therefore their incorporation into ILVs. These sorting processes are regulated by various Rab GTPases. Once matured, MVEs that are not targeted to lysosomes or autophagosomes for degradation are transported to the plasma membrane on microtubules. At this

step, docking and fusion are the two final processes required for exosome release. Rabs, actin and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins are involved in these steps of exosome release. In the case of microvesicle biogenesis, endocytic uptake (dashed arrow) and recycling will respectively decrease and increase the targeting of membrane (and membrane bound) cargoes to microvesicles. Of note, as the release of exosomes requires tightly regulated steps of transport, tethering and fusion of MVE to plasma membrane (apart from cargo sorting) this could account for the time difference between the generation and release of both types of extracellular vesicles. OK * denotes proteins of *Caenorhabditis elegans*.

Figure 5: Fate of extracellular vesicles in recipient cells.

In recipient cell (which can be the producing cell itself) exogenous extracellular vesicles will bind to the cell surface (see inset) and can follow various fates. Depending on the cell type they can remain bound to the surface (for example to integrins) and can initiate intracellular signalling pathways (for example antigen presentation). Extracellular vesicles may also be internalized by multiple routes. Internalization will target exogenous extracellular vesicles into the canonical endosomal pathway, whereby they reach multivesicular endosomes (MVEs) where the uptaken vesicles will likely mix with endogenous intraluminal vesicles (ILVs). Fusion of MVEs with the lysosome will lead to the degradation of extracellular vesicles and the recycling of their content to fuel recipient cell metabolism. Extracellular vesicles either docked at the plasma membrane or at the limiting membrane of MVEs can release their intraluminal content into the cytoplasm of the recipient cell by fusion, a process which is so far poorly understood but of major importance for delivery of intraluminal cargoes such as miRNA. Of note, there is no evidence so far to exclude potential recycling to the plasma membrane of endocytosed vesicles (dashed arrows). ECM, extracellular matrix; ICAM, intercellular adhesion molecules; TIM4, T-cell immunoglobulin mucin receptor 4.

Glossary

reticulocytes: precursors of red blood cells (erythrocytes)

sorting machineries: protein complexes mediating cargo sorting in endosomes

major histocompatibility complex: a group of genes that code for cell-surface glycoproteins that help the immune system to determine self and nonself

syntenin: an intracellular adaptor protein linking syndecan-mediated signalling to the cytoskeleton

syndecan: a single transmembrane domain heparan sulfate proteoglycan that binds a large variety of ligands, such as growth factors and fibronectin among others

ceramide: a lipid molecule composed of sphingosine and a fatty acid linked through an amide bound; in fact, many chemically diverse ceramides have been described, showing that ceramide is not a single molecular species but rather a family of related molecules.

sphingomyelin: a type of sphingolipid found in animal cell membranes.

tetraspanin family: family of proteins with four transmembrane domains which allow association with other members of the family and with other proteins to generate dynamic membrane domains.

GlycosylPhosphatidylnostol (GPI)-anchored proteins: a posttranslational modification, comprising a phosphoethanolamine linker, glycan core, and phospholipid tail, that anchors the modified protein to the outer leaflet of the cell membrane.

lipid rafts: specialized membrane microdomains enriched in cholesterol and glycosphingolipid which serve as organizing centers for the assembly of signalling molecules.

KRAS–MEK signalling pathway: interaction between the proto-oncogene KRAS which encodes a small GTPase and the downstream effector, the canonical RAF–MEK–EERK signalling pathway. Both pathways have roles in cell division, cell differentiation and apoptosis.

Major Vault protein: the main component of ribonucleoparticles termed vaults, which also contains two additional proteins, the vault poly (ADP-ribose) polymerase (vPARP) and the telomerase-associated protein 1 (TEP1), and several short untranslated, vault RNAs (vRNA). It has been implicated in the regulation of several cellular processes including transport mechanisms, signal transmissions and immune responses.

Y-box protein 1: a transcription factor shown to have a role in oncogenic cell transformation, multiple drug resistance and dissemination of tumours.

aminophospholipid translocases: enzymes which transport phosphatidylserine and phosphatidylethanolamine from one side of a bilayer to another.

scramblases: proteins responsible for the translocation of phospholipids between the inner and outer leaflet of a cell membrane.

calpain: a calcium-dependent protein expressed ubiquitously in mammals and many other organisms.

Rho family of small GTPases: a family of small signalling G proteins implicated in the regulation of many aspects of actin dynamics.

brush border: the microvilli-covered surface of epithelial cells found in enterocytes in the intestine.

Warburg effect: an aerobic process whereby cancer cells produce energy by a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than by oxidation of pyruvate in mitochondria

zipcode RNA sequence motifs: cis-acting regulatory sequences (25 nucleotides) in the 3′-untranslated region (3′UTR) of mRNA transcripts that mediate binding of a ribonuclear protein complex to the mRNA, thereby temporarily blocking mRNA translation and that mediate movement of mRNA via the cytoskeleton to a cellular location where mRNA is released from protein binding and translation initiates.

immunological synapse: a specialized cell–cell junction between a thymus-derived lymphocyte (T cell) and an antigen-presenting cell.

Hedgehog: an essential signalling molecule, termed morphogen, required for numerous processes during animal development.

ISGylation: an ubiquitin-like modification that controls exosome release by decreasing the number of multivesicular endosomes.

caveolin: the principal component of caveolae, which are involved in receptor (clathrin)-independent endocytosis, mechanotransduction and lipid homeostasis.OK

SNARE proteins: an acronym derived from "**SNA**P (Soluble NSF Attachment Protein) **RE**ceptor"); their primary role is to mediate the fusion of intracellular vesicles with their target membrane bound compartments.

Synaptotagmin family: family of membrane-trafficking proteins that has been implicated in calcium- dependent neurotransmitter release.

Protein kinase C: a serine/threonine kinase which plays important roles in several signal transduction cascades by controlling the function of other proteins through their phosphorylation.

$P2x_7$ receptors: trimeric ATP-gated cation channels found predominantly, but not exclusively, on immune cells, which have been implicated in various inflammatory, immune, neurologic and musculoskeletal disorders.

follicular dendritic cells: cells of the immune system found in primary and secondary lymph follicles of the B cell areas of the lymphoid tissue.

lectin: a carbohydrate-binding protein that is highly specific for sugar moieties.

proteoglycans: heavily glycosylated proteins consisting of a “core protein” with one or more covalently attached glycosaminoglycans (GAG) chains

Intercellular adhesion molecules (ICAMs): member of the immunoglobulin superfamily, which are involved in inflammation, immune responses and in intracellular signalling events.

macropinocytosis a form of regulated endocytosis that involves the non-specific uptake of extracellular material (such as small soluble molecules, nutrients, antigens) by invagination of the plasma membrane, which is then pinched resulting in small vesicles in the cytoplasm.

trophoblast: cells which form the outer layer of a blastocyst, provide nutrients to the embryo and give rise to a large part of the placenta.

microglia: a type of brain glial cell acting as the first and main endogenous immune defense in the central nervous system

astrocytes: star-shaped glial cells in the brain involved in nutrient supply, maintenance of extracellular ion balance and in tissue repair following brain injuries

filopodia: highly dynamic actin-rich cell-surface protrusions used by cells to sense their external environment.

suicide mRNA/protein: suicide gene which encodes nonmammalian enzymes that convert an inactive drug into highly toxic metabolites that inhibit the synthesis of nucleic acids and causes cells to go to apotosis

Key points

- Secretion of extracellular vesicles was initially described as means of selective elimination of proteins, lipids and RNA from the cells. Now, extracellular vesicles are also considered as a new mode of intercellular communication.
- In any given setting, population of extracellular vesicles comprises diverse subpopulations that can differ in size, morphology, composition or biogenesis mechanisms. Complementary methods of analysis are required to distinguish between these subpopulations.
- Several machineries, prominently including components of the endocytic sorting machineries, act concomitantly for the generation of extracellular vesicles. In result, extracellular vesicles can largely vary in terms of their composition and may carry specific sets of proteins, lipids, RNA species that then determine their fate and functions.
- Generation of extracellular vesicles requires a fine tuning of various intracellular trafficking processes, which define the composition of nascent vesicles and impact their generation and, in the case of exosomes, their secretion from an intracellular compartment.

- Interactions of extracellular vesicles with recipient cells ~~and their~~ can have various effects on the target cell, from stimulating signalling pathways to providing trophic support, which depends on the mode of interaction and the intracellular fate of the vesicles in case of their uptake.
- Studies of the cell biology of extracellular vesicles is not only essential for addressing cell biological questions but is also critical to open new avenues for their clinical use as biomarkers, cargo vehicles for targeted delivery of compounds or as specific modulators of cell behaviours.

Author biographies

Guillaume van Niel, is a research director at CNRS and team leader of the Center of Psychiatry and Neurosciences, U894 INSERM. His research focuses on the processes and functions of endosomes and exosomes with implications in neuropathies using Zebrafish as an *in vivo* model system.

Gisela D'Angelo, is a research associated at CNRS, in Graça Raposo team, UMR144, at the Curie Institut. Her research interest is on the biogenesis and function of extracellular vesicles in developmental biology and cancer using Drosophila epithelium as an *in vivo* model system.

Graça Raposo, is a research director at CNRS, Deputy Director and team leader of the Department of Cell Biology, UMR144, in Institut Curie. Her research interest is on Intracellular trafficking, the biogenesis and functions of exosomes and lysosome related organelles such as melanosomes with implications lysosomal diseases and cancer.

Author contributions

All authors contributed equally to all aspects of the article (researching data for article, substantial contribution to discussion of content, writing, review/editing of manuscript before submission).

Competing interests statement

The authors declare no competing interests.

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