Shedding light on the cell biology of extracellular vesicles

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Abstract | Extracellular vesicles are a heterogeneous group of cell-derived membranous structures comprising exosomes and microvesicles, which originate from the endosomal system or which 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 genetic material. Knowledge of the cellular processes that govern extracellular vesicle biology is essential to shed light on the physiological and pathological functions of these vesicles as well as on clinical applications involving their use and/or analysis. However, in this expanding field, much remains unknown regarding the origin, biogenesis, secretion, targeting and fate of these vesicles.

Reticulocytes
Precursors of red blood cells (erythrocytes).

Apart from the release of secretory vesicles by specialized cells, which carry, for example, hormones or neurotransmitters, all cells are capable of secreting various types of membrane vesicles, known as extracellular vesicles, and this process is conserved throughout evolution from bacteria to humans and plants[1–3]. The secretion of extracellular vesicles was initially described as a means of eliminating unneeded compounds from the cell[4]. However, we now know that extracellular vesicles are more than just 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[5–7].

Even though the 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 their characterization and manipulation of their properties and functions. Insights into the biogenesis of secreted vesicles were 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 for vesicles of an unknown origin released from a variety of cultured cells and carrying 5′-nucleotidase activity[12]. Subsequently, the term exosome was adopted to refer to membrane vesicles (30–100 nm in diameter) released by reticulocytes 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-1990s, exosomes were reported to be secreted by B lymphocytes[15] and dendritic cells[16] with potential functions related to immune regulation and were considered for use as vehicles in antitumoural immune responses. Exosome secretion has now been extended to many different cell types, and its implications in intercellular communication in normal and pathological states are now well documented[5].

Microvesicles, formerly called 'platelet dust', were first 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 have been studied mainly for their role in blood coagulation[19,20], more recently, they were reported to have a role in cell–cell communication in various cell types, including cancer cells[21], where they are generally called oncosomes. Microvesicles range in size from 50 nm to 1,000 nm in diameter but can be even larger (up to 10 μm) 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

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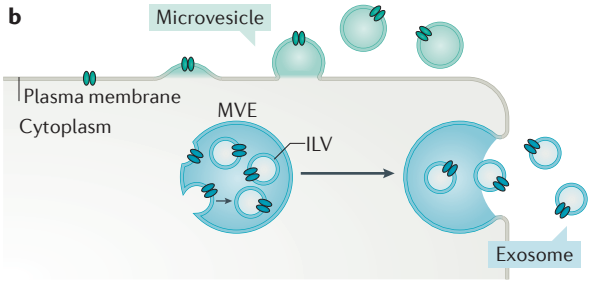
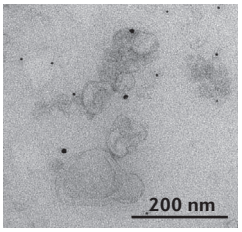
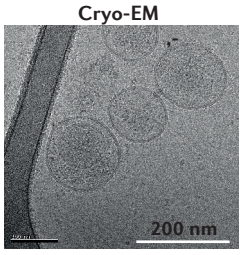
a

	Exosomes	Microvesicles
Origin	Endosome	Plasma membrane
Size	50–150 nm	50–500 nm (up to 1 µm)
Other names (according to their origin, size and morphology)	• Prostasomes • Tolerosomes • Dexosomes • Nanovesicles • Exosome-like vesicles and others	• Microparticles • Blebbing vesicles • Shedding vesicles • Oncosomes • ARRMs • Migrasomes • Neurospheres • Apoptotic bodies







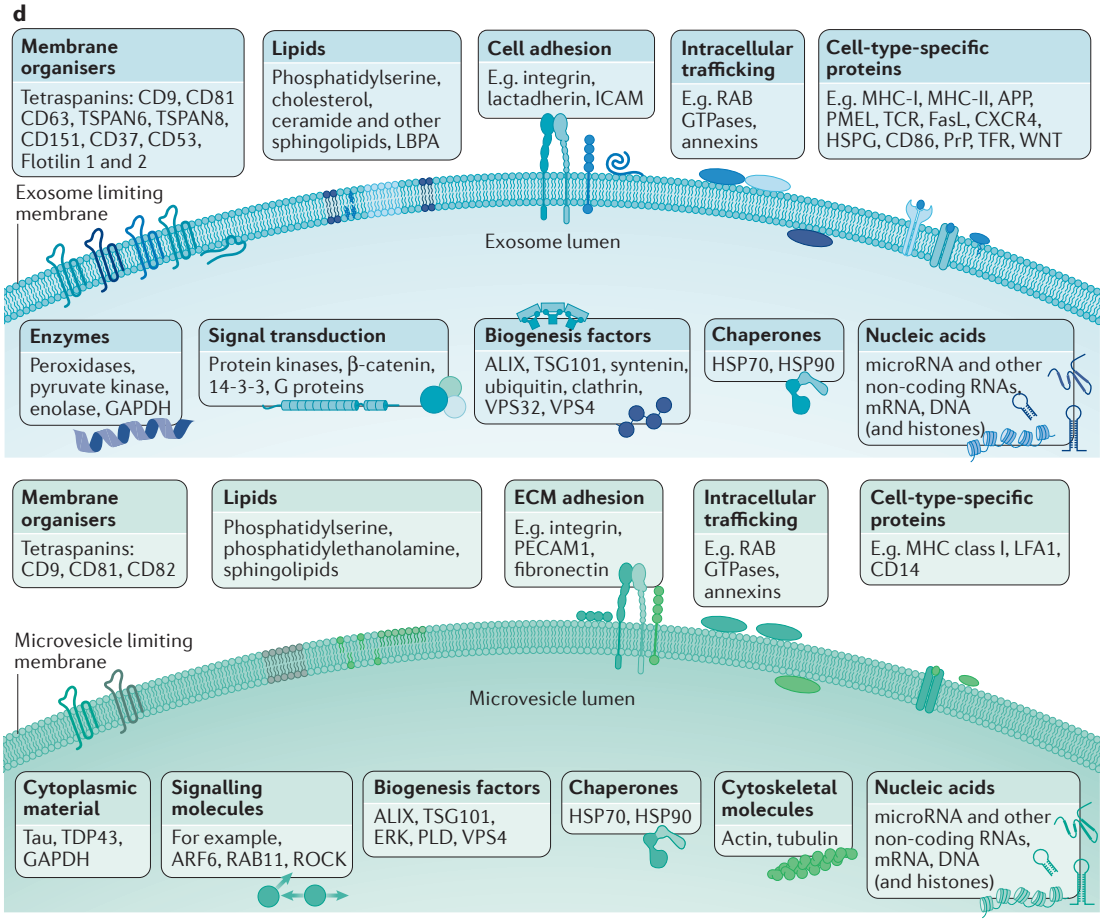


Figure 1 | **Main features of extracellular vesicles. a** | Extracellular vesicles comprise a heterogeneous population of membrane vesicles of various origins. Their size may vary (typically between 50 nm and 500 nm, but they can be even larger, measuring 1–10 µm). Over the past two decades, extracellular vesicles have been named based on 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, in which case they 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). However, when observed in a close-to-native state by cryo-electron microscopy (cryo-EM), they appear as round structures enclosed by double-leaflet membranes (bottom panel). **d** | Study of extracellular vesicle composition revealed that they can carry various cargoes, including proteins, lipids and nucleic acids, and this content can vary widely 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, depending on 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 a 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. ALIX, ALG-2 interacting protein X; APP, amyloid precursor protein; ARF6, ADP-ribosylation factor 6; ARMMs, arrestin-domain-containing protein 1-mediated microvesicles; CXCR4, CXC-chemokine receptor 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP70, heat shock 70 kDa protein; HSPG, heparan sulfate proteoglycan; ICAM, intercellular adhesion molecule; LBPA, lyso-bis-phosphatidyl acid; LFA1, lymphocyte function-associated antigen 1; MHC, major histocompatibility complex; PECAM1, platelet endothelial cell adhesion molecule; PLD, phospholipase D; PrP, prion protein; ROCK, RHO-associated protein kinase; TCR, T cell receptor; TDP43, TAR DNA-binding protein 43; TFR, transferrin receptor; TSG101, tumour susceptibility gene 101 protein; TSPAN, tetraspanin; VPS, vacuolar protein sorting-associated protein. Images in part **c** courtesy of Roberta Palmulli (G. Raposo's laboratory, URM144, Institut Curie) for conventional TEM and Daniel Levy (UMR168, Institut Curie, France) for cryo-EM.

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state and releases 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 than on 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 biopsy samples 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 apoptotic bodies; migrasomes, which transport multivesicular cytoplasmic content during cell migration[24]; and arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs)[25], which are largely uniform microvesicles of ~50 nm in diameter that have been shown to bud directly from the plasma membrane in a manner resembling the budding of viruses and dependent on ARRDC1 and on endosomal sorting complex required for transport (ESCRT) proteins (similarly to a subpopulation of exosomes; see below).

The overlapping range of size, similar morphology and variable composition challenge current attempts to devise a more precise nomenclature for extracellular vesicles[26,27]. Nevertheless, novel isolation and characterization methods are being developed to allow a more thorough description of the respective functions of the different types of extracellular vesicles and to establish a suitable classification and terminology. Moreover, to validate the 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.

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 the 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 primarily generate extracellular vesicles from the cell surface with characteristics of exosomes, probably by exploiting molecular components and mechanisms at the plasma membrane 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 the generation of microvesicles and exosomes occurs at distinct sites within the cell, common intracellular mechanisms and sorting machineries are involved in the biogenesis of both entities. In many cases, these shared mechanisms hinder the possibility of distinguishing between the different vesicle subpopulations[5]. Mechanistic details of extracellular vesicle biogenesis have only now 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 the 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).

Cargoes and their targeting to the site of extracellular vesicle generation. The nature and abundance of extracellular vesicle cargoes[30] (FIG. 1d) are cell-type-specific and are 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 the expression of the major histocompatibility complex (MHC) class II[32], promotes MVE formation with a consequent release of extracellular vesicles, probably 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]. Hence, cargoes that are preferentially recycled to the plasma membrane are not likely to be 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, the protein syntenin, by acting both in the recycling[36] and in the sorting of syndecan in MVEs[37] for exosome biogenesis, seems to be a potential regulator of the crosstalk 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; also known as MMP14) and the vesicular SNARE (v-SNARE) vesicle-associated membrane protein 3 (VAMP3), into tumour-derived microvesicles[38,39]. In addition to ARF6-regulated endosomal trafficking, VAMP3 mediates the trafficking and incorporation of MT1-MMP into tumour-derived microvesicles in a CD9-dependent manner. This suggests that VAMP3-positive and ARF6-positive recycling

Sorting machineries
Protein complexes mediating cargo sorting in endosomes.

Major histocompatibility complex
(MHC). A group of genes that code for cell surface glycoproteins that help the immune system to determine self and non-self.

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.

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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 RAS-related protein RAB22A colocalizes with budding microvesicles and mediates packaging and loading of cargo proteins in hypoxic breast cancer cells[40].

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 in uncovering the mechanisms involved in the formation of MVEs and ILVs[41]. The ESCRT machinery acts in a stepwise manner wherein ESCRT-0 and ESCRT-I subunits cluster ubiquitylated 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; also known as HGS) 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

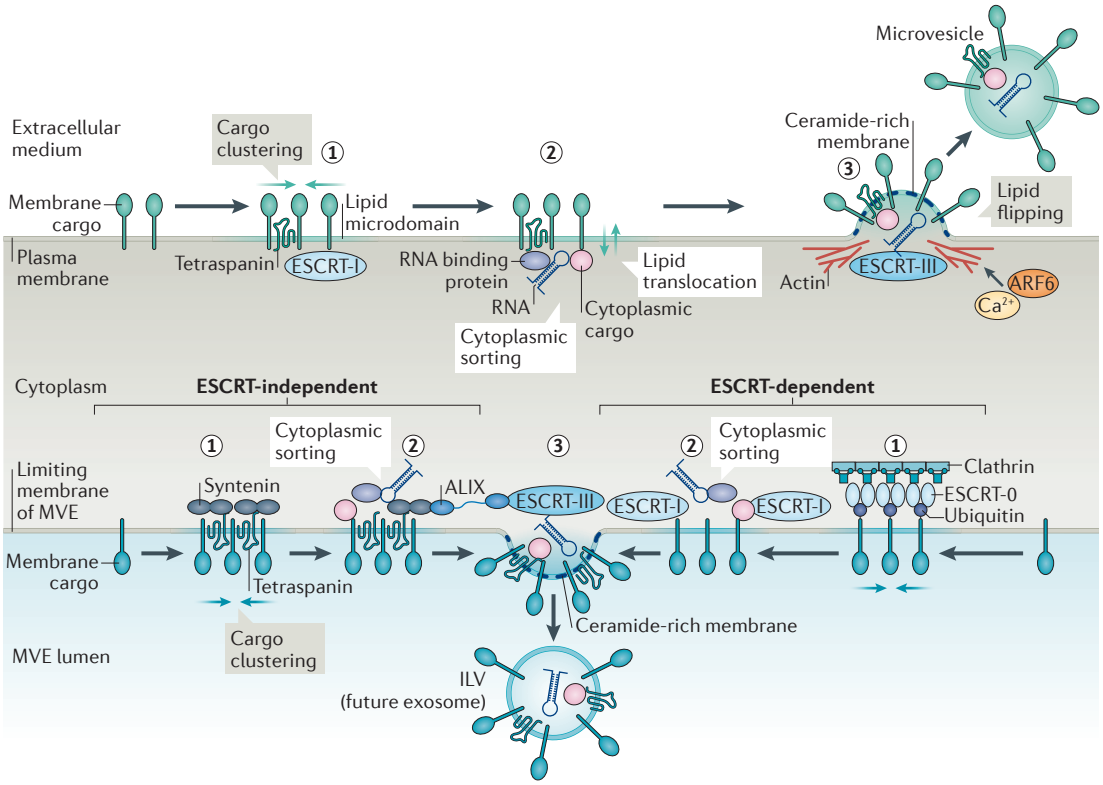

Figure 2 | **Biogenesis of extracellular vesicles.** Several sorting machineries are involved in the different steps required for generating exosomes and microvesicles. First, lipids and membrane-associated proteins are clustered in discrete membrane microdomains of the plasma membrane for microvesicles (top) and of the limiting membrane of the multivesicular endosome (MVE) for exosomes (bottom) (step 1). Such microdomains certainly also participate in the recruitment of soluble components, such as cytosolic proteins and RNA species, that are fated for sorting in extracellular vesicles (step 2). Altogether, formation of these clustered microdomains together with additional machineries promotes 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 the MVE (step 3). Transmembrane proteins sorted on exosomes and microvesicles keep the same topology as at the plasma membrane. Mechanisms of exosome biogenesis are fairly well understood and importantly involve subunits of endosomal sorting complex required for transport (ESCRT), although to different degrees — ESCRT-III is required for the scission of the intraluminal vesicles (ILVs) into the MVE lumen, but cargo clustering and membrane budding can occur by either ESCRT-dependent or ESCRT-independent mechanisms. The mechanisms involved in the biogenesis of microvesicles are still being revealed. Notably, the molecular machineries that act at the different steps of extracellular vesicle biogenesis are partly common to exosomes and microvesicles (including ESCRT proteins and the generation of ceramide by sphingomyelinase). One exception is the flipping of specific lipid species between the leaflets of the budding membrane, which has been uniquely reported for 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 the biogenesis of these vesicles. ALIX, ALG-2 interacting protein X; ARF6, ADP-ribosylation factor 6.

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components. A medium-throughput RNA interference screen targeting[23] multiple components of the ESCRT machinery and associated proteins has revealed various roles for selected members of this family in exosome generation. Their inactivation affects 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 the ESCRT accessory protein ALG-2 interacting protein X (ALIX; also known as programmed cell death 6-interacting protein), which bridge cargoes and the ESCRT-III subunit vacuolar protein sorting-associated protein 32 (VPS32; also known as CHMP4)[37].

Exosomes can also be 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 by neutral type II sphingomyelinase, which hydrolyses sphingomyelin to ceramide[45]. Ceramide may then allow the generation of membrane subdomains[46], which impose a 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 have been shown to regulate 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 patients with Down syndrome[51]. The tetraspanins CD81, CD82 and CD9 are also directly involved in the sorting of various cargoes to exosomes[52,53]. Mechanistically, these proteins form clusters and dynamic membrane platforms with other tetraspanins and with different transmembrane and cytosolic proteins[54] probably 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 to be 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). However, tetraspanins also regulate the intracellular routing of cargoes, such as integrins[56], towards MVEs, which indicates that impairment of their function may affect different steps of exosome generation. Thus, it seems that both ESCRT-dependent and ESCRT-independent mechanisms operate in exosome biogenesis, and their contributions 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 result from co-sorting with other proteins, such as the chaperones heat shock 70 kDa protein (HSP70) and heat shock cognate 71 kDa protein (HSC70), which are found in exosomes derived from most cell types[57,58]. Membrane cargoes, such as glycosylphosphatidylinositol (GPI)-anchored proteins are present in exosomes, probably because of their affinity for lipid domains and lipid rafts 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 microRNAs (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 RNA-binding proteins in the membrane subdomains[69] or the miRNA-induced silencing complex (miRISC) and protein argonaute 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 acting through AGO2 (REF. 71), major vault protein[72] and Y-box-binding protein 1 (also known as YBX1)[73].

In sum, exosome biogenesis is certainly complex, 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, MHC class II, which can be targeted to MVEs by both ESCRT-dependent and ESCRT-independent mechanisms)[52,77], or on different maturation products of the cargo (as is the case for the melanocyte protein PMEL, for which the luminal domain, which is generated by proteolysis, is sorted by ESCRT-independent mechanisms, whereas the transmembrane domain is sorted by an ESCRT-dependent

Ceramide
A lipid molecule composed of sphingosine and a fatty acid linked through an amide bond; 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
A family of proteins with four transmembrane domains that allow association with other members of the family and with other proteins to generate dynamic membrane domains.

Glycosylphosphatidylinositol (GPI)-anchored proteins
Proteins with a post-translational modification comprising a phosphoethanolamine linker, a glycan core and a phospholipid tail. This modification anchors the protein to the outer leaflet of the cell membrane.

Lipid rafts
Specialized membrane microdomains enriched in cholesterol and glycosphingolipid that serve as organizing centres for the assembly of signalling molecules.

KRAS–MEK signalling pathway
The interaction between the proto-oncogene *KRAS*, which encodes a small GTPase, and its downstream effector, the canonical RAF proto-oncogene serine/threonine-protein kinase (RAF)–MEK–ERK 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 contain two additional proteins, the vault poly(ADP-ribose) polymerase (vPARP) and telomerase-associated protein 1 (TEP1), and several short, untranslated vault RNAs (vRNAs). It has been implicated in the regulation of several cellular processes, including transport mechanisms, signal transmission and immune responses.

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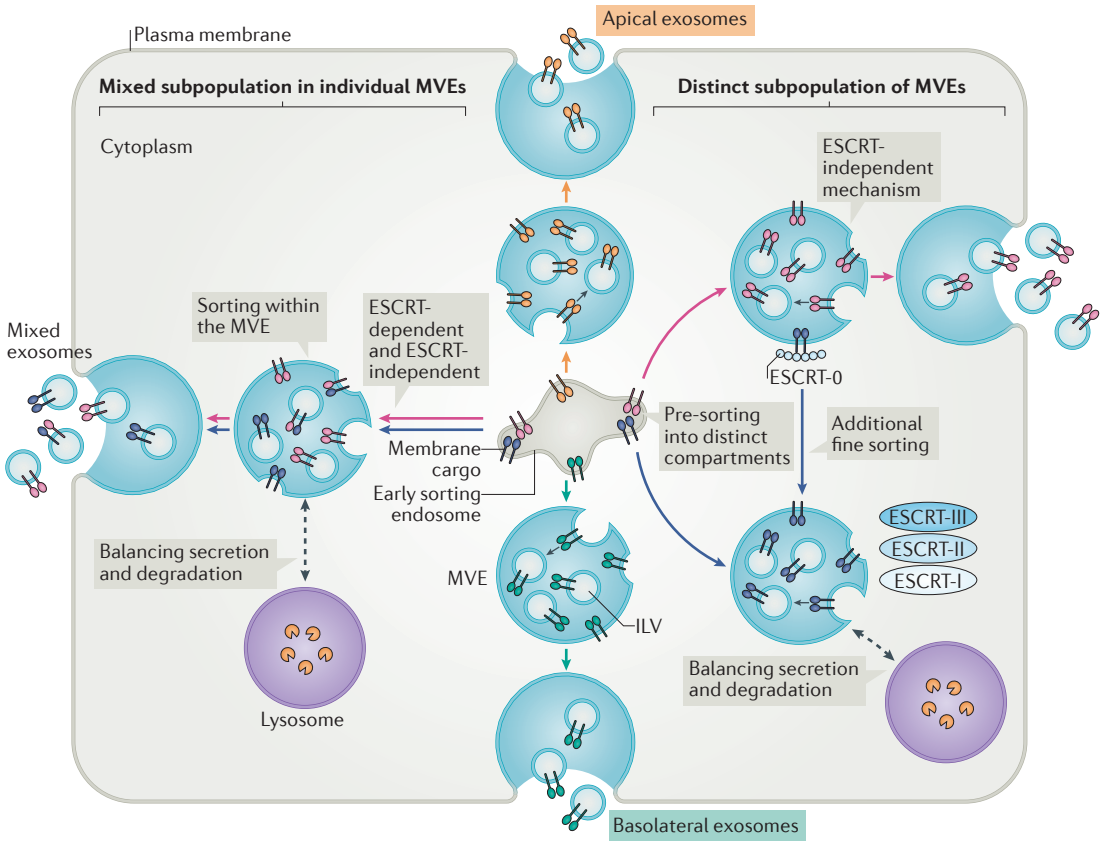

Figure 3 | **Origin of exosome diversity in relation to sorting machineries.** The diversity of extracellular vesicle subpopulations with distinct composition and function that are generated by a given cell type is often attributed to the production of distinct sets of exosomes and microvesicles[23,192]. This diversity is well exemplified by the secretion of different exosome subpopulations — with distinct morphology and composition — from the apical and basolateral domains of polarized cells such as intestinal epithelial cells[193–195]. Such exosome subpopulations probably originate from subpopulations of multivesicular endosomes (MVEs). The distinct compositions of these subpopulations reflect 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 subpopulations of MVEs (see right-hand side) or concomitantly in single MVEs to generate distinct subpopulations of intraluminal vesicles (ILVs) (see left-hand side). The reality is most likely a mix of these two possibilities, with distinct contributions 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 or ubiquitylation)[49,193] or potentially on the stage of maturation of the 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.

Y-Box-binding protein 1
A transcription factor shown to have a role in oncogenic cell transformation, multidrug resistance and the dissemination of tumours.

Aminophospholipid translocases
Enzymes that transport phosphatidylserine and phosphatidylethanolamine from one side of a bilayer to the other.

Scramblases
Proteins responsible for the translocation of phospholipids between the inner and outer leaflets of a cell membrane.

Calpain
A calcium-dependent protein expressed ubiquitously in mammals and many other organisms.

mechanism)[49]. Therefore, several mechanisms could act concomitantly or sequentially 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, these data support a model whereby the biogenesis of exosomes involves several distinct mechanisms for the preferential recruitment of cargoes probably generating heterogeneous populations of ILVs and exosomes within common or distinct subpopulations of MVEs[5,6]. Overall, as major regulators of the composition of exosomes, endosomal sorting machineries seem to be 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.

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 recently started to emerge. 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 (flippases and floppases), scramblases and calpain 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).

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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 that they form, contribute to microvesicle biogenesis. One important lipid component is cholesterol, which is abundant in microvesicles and the pharmacological depletion of which impairs microvesicle generation in activated neutrophils[83].

In addition to lipids, cytoskeletal elements and their regulators are certainly required for microvesicle biogenesis. The activity of the RHO family of small GTPases and of the RHO-associated protein kinase (ROCK), which are important regulators of actin dynamics, induces microvesicle biogenesis in different populations of tumour cells[84]. As another example, in the enterocyte brush border, 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 tumour-derived microvesicles (oncosomes) is also tightly associated with metabolic changes, the so-called Warburg effect[86]. In breast cancer cells, elevated glutaminase activity relies on microvesicle secretion and is dependent on RHO GTPases[87], inhibition of which blocks microvesicle biogenesis. This suggests that formation and loading of microvesicles are 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, which 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 the cell surface. One possible mechanism revealed from studies of cancer cells suggests the involvement of conserved zipcode RNA sequence motifs in the 3′ untranslated regions in mRNA targeting into microvesicles[90], but the details of this process remain to be discovered.

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 for both types of extracellular vesicles. The release of microvesicles would probably be 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. By contrast, the release of exosomes requires additional steps to sort cargoes to MVEs, then to ILVs and extra steps to target MVEs to the plasma membrane and to prime them for secretion. Such differences could be relevant from a functional point of view as they impose additional regulatory checkpoints for the secretion of exosomes compared with microvesicles. Whereas the release may be constitutive in some cases, such as embryonic development, cell differentiation and in general during maintenance of physiological homeostasis, this process may also be subjected to further modulation by the physiological state of the cell and by 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 synapses[52,91]. As the release of microvesicles is likely to be 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.

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 release (FIGS 3,4). The regulation of the balance between degradative and secretory capacity of MVEs remains largely unexplored, but the establishment of this balance undoubtedly affects 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 in exosome release[94,95] and, for example, has been shown to trigger apical secretion of Hedgehog-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 probably imposed by the sorting machineries at MVEs. While the different components of the ESCRT machinery have various effects on exosomes secretion[23] and are generally associated with degradative MVEs, the syndecan–syntenin–ALIX pathway seems to be restricted so far to exosome secretion[37]. Along the same lines, MHC class II is targeted to MVEs fated for lysosomal degradation through ubiquitylation (probably recruiting ESCRT machinery) while ubiquitin-independent (and probably ESCRT-independent) mechanisms target MHC class 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), which is subject to ISGylation that

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 microvillus-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 the 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 synapses
Specialized cell–cell junctions between a thymus-derived lymphocyte (T cell) and an antigen-presenting cell.

Hedgehog
An essential signalling molecule, termed a morphogen, required for numerous processes during animal development.

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

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Caveolin
The principal component of caveolae, which are involved in receptor (clathrin)-independent endocytosis, mechanotransduction and lipid homeostasis.

favours its lysosomal degradation (and thereby impairment of exosome secretion)[94], or tetraspanin 6 (REF. 97), overexpression of which slows lysosomal degradation probably 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 lysosomal 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 the 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 that it does so by interacting with caveolin 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 counterbalanced by their fusion with the autophagosome. Autophagosomes and MVEs can both secrete their contents, but the molecular mechanisms regulating these secretory pathways are likely to be distinct.

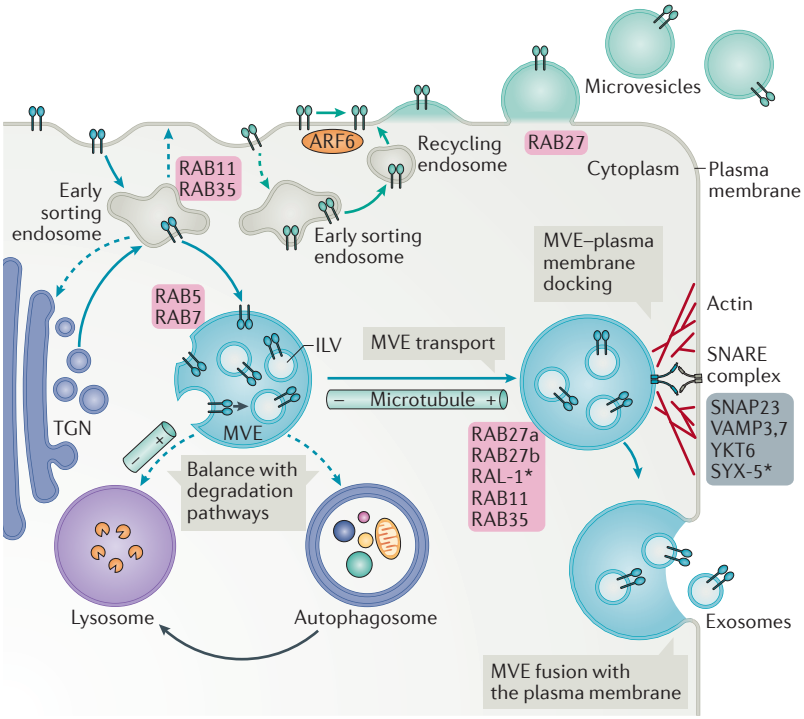

Figure 4 | **Interdependency of intracellular trafficking routes in the generation of extracellular vesicles.** The generation of exosomes and microvesicles requires tuned regulation of multiple intracellular trafficking steps (blue arrows for exosomes, green arrows for microvesicles) that influence the targeting of cargoes to the site of extracellular vesicle biogenesis and, 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 the MVE (dashed arrows) and therefore their incorporation into intraluminal vesicles (ILVs). These sorting processes are regulated by various RAS-related protein (RAB) GTPases. Once matured, MVEs that are not targeted to lysosomes or autophagosomes for degradation are transported along microtubules to the plasma membrane. At this step, docking and fusion are the two final processes required for exosome release. RABs, actin and 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 the plasma membrane (apart from cargo sorting), this could account for the time difference between the generation and release of the two types of extracellular vesicles. ARF6, ADP-ribosylation factor 6; RAL-1, RAL (Ras-related GTPase) homolog; SNAP23, synaptosomal-associated protein 23; SYX-5, syntaxin 5; VAMP3, vesicle-associated membrane protein 3. *Denotes *Caenorhabditis elegans* proteins.

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 cytoskeleton (actin and microtubules), associated molecular motors (dynein, kinesins and 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 synapses, 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 the transport of MVEs towards lysosomes. Targeting to lysosomes occurs by retrograde transport on microtubules (towards microtubule minus ends), and the 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 required for the release of exosomes[37]. These dual effects on exosome secretion seem 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 the 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, is likely to 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

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the motility of MVEs towards the plasma membrane, and both RAB27 isoforms act on the step following MVE transport, which 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 the secretion of secretory lysosomes, the so called lysosome-related organelles[109], which indicates 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 the reported involvement of additional RABs and their effectors, such as effectors of RAB11 and RAB35 (REFS 110,111), in the direct regulation or the potential priming of MVE secretion.

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 probably mediated by SNARE proteins and synaptotagmin family members[112]. A SNARE complex known to be involved 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 (REF. 113). This complex is involved in exosome secretion in some cells (the human leukaemia cell line K562)[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+} (REFS 116–118), which may have a role in the activation of the SNARE complexes. The implication of synaptosomal-associated protein 23 (SNAP23) — a SNARE shown to regulate secretion of lysosome-related organelles from 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 the synaptobrevin homologue Ykt6 (REF. 121) in *Drosophila*, syntaxin 5 in *C. elegans*[122] and syntaxin 1a[123] in mammals, again reflect the diversity of regulators that could be involved in exosome secretion, most likely depending on the organism, the cell type or the MVE subtype. 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 by these perturbations in the producing cells. 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.

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 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 the ATPase VPS4, mostly involved in exosome generation as part of the ESCRT machinery, were reported to participate in the scission and release of ARMMs[25]. Shedding of ESCRT-dependent microvesicles was also reported in *C. elegans* embryos upon loss of TAT-5, the conserved flippase P4-ATPase, 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]. It is known that strong microvesicle release is induced by increased Ca^{2+} concentration, which by activating scramblase and calpain leads to a loss of membrane phospholipid asymmetry and the reorganization of the cytoskeleton (see above) or through the activation of protein kinase C by phorbol esters[130]. Release of microvesicles has also been shown to depend on ATP-mediated activation of $P2X_7$ receptors, 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.

Targeting to recipient cells

Once released into the extracellular space, extracellular vesicles can reach recipient cells and deliver their contents to elicit functional responses and promote phenotypic changes that will affect 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

SNARE proteins
Proteins named from SNAP (soluble NSF attachment protein) receptor; their primary role is to mediate the fusion of intracellular vesicles with their target membrane-bound compartments.

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

Protein kinase C
A serine/threonine kinase that 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; these receptors have been implicated in various inflammatory, immune, neurological and musculoskeletal disorders.

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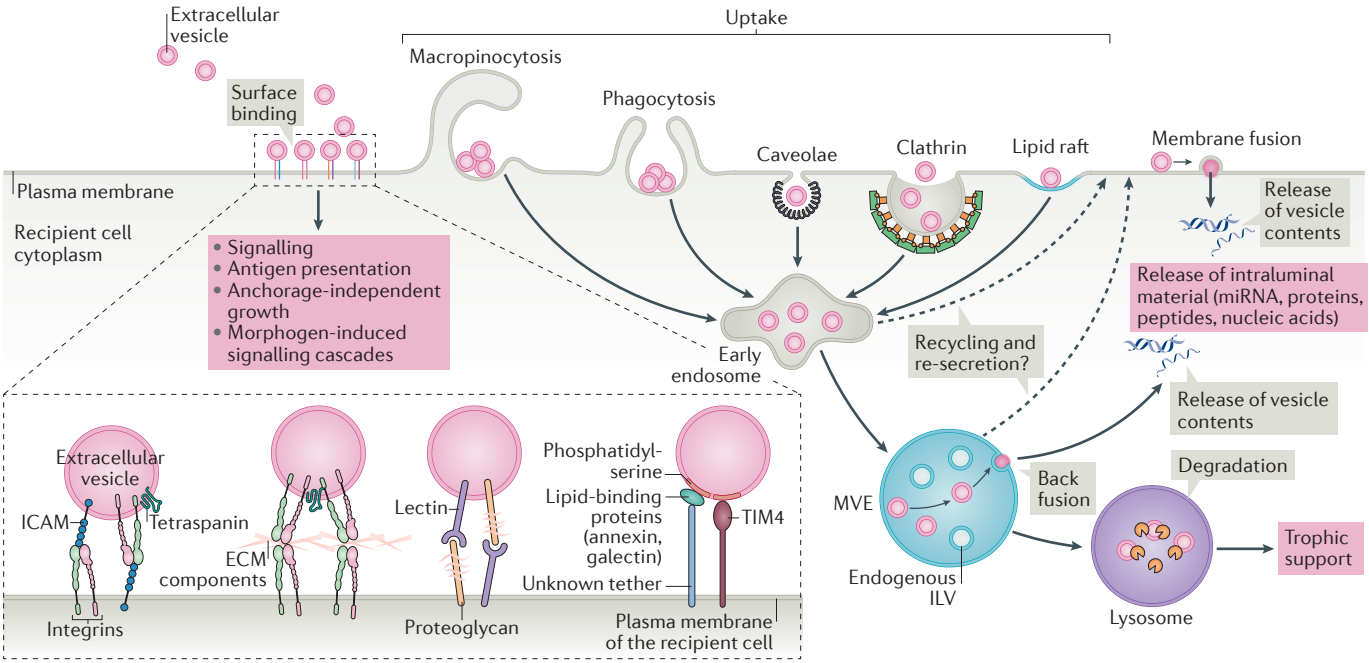

Figure 5 | **Fate of extracellular vesicles in recipient cells.** In the recipient cell (which can be the producing cell itself), exogenous extracellular vesicles will bind to the cell surface (see inset) and can undergo various fates. Depending on the cell type, they can remain bound to the surface (for example, to integrins) and can initiate intracellular signalling pathways (for example, antigen presentation). Extracellular vesicles may also be internalized by multiple pathways. Internalization will target exogenous extracellular vesicles into the canonical endosomal pathway, whereby they reach multivesicular endosomes (MVEs), in which the internalized vesicles are likely to 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 contents to fuel the metabolism of the recipient cell. Extracellular vesicles docked either at the plasma membrane or at the limiting membrane of MVEs can release their intraluminal contents into the cytoplasm of the recipient cell by fusion, a process that is currently poorly understood but of major importance for delivery of intraluminal cargoes such as microRNA (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 molecule; TIM4, T cell immunoglobulin mucin receptor 4.

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, and they 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 contents and sizes, the principles of uptake and general intercellular trafficking of different subpopulations of extracellular vesicle are likely to be shared.

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 in follicular dendritic cells[135], intestinal epithelial cells[136], dendritic cells[137], or neurons[138] and also in liver, lungs or lymph nodes[136,139]. Of note, the recipient cell can also be the producing cell itself, generating autocrine responses[124].

Several mediators of these interactions are known, including tetraspanins, integrins, lipids, lectins, heparan sulfate proteoglycans 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 are available. For example, integrins on extracellular vesicles can interact with adhesion molecules such as intercellular adhesion molecules (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 composition[139]. Exosomal tetraspanins could also regulate cell targeting. They have been shown to interact with integrins[144] and to promote exosome docking and uptake by selected recipient cells[145,146]. Other molecules such as heparan sulfate proteoglycans and lectins, both present in extracellular vesicles and at the plasma membrane, contribute to the docking and/or attachment of these vesicles to recipient cells (FIG. 5). Glypican 1, a cell surface proteoglycan that bears heparan sulfate, and CD44, a cell surface glycoprotein involved in cell–cell interactions, are involved in exosome[147] and microvesicle[148] docking, respectively. The lipid composition of extracellular vesicles can also have an impact on recipient-cell targeting.

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

Lectins
Carbohydrate-binding proteins that are highly specific for sugar moieties.

Proteoglycans
Heavily glycosylated proteins consisting of a 'core protein' with one or more covalently attached glycosaminoglycan (GAG) chains.

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

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For example, phosphatidylserine can recruit specific lipid-binding proteins such as galectin 5 or annexin 5 (REFS 140,149,150) that then induce docking of vesicles to the target cell membrane.

Uptake and intracellular fate of extracellular vesicles. Once they have bound to recipient cells, extracellular vesicles may remain at the plasma membrane[52,135] or may be internalized by clathrin-mediated or clathrin-independent endocytosis, such as macropinocytosis and phagocytosis[151–153] as well as through endocytosis via caveolae and lipid rafts[154–156] (FIG. 5). Of note, certain cell types, such as HeLa cells or Epstein–Barr virus (EBV)-transformed B cell lines release clusters of exosomes, as a result of tethering the vesicles by the protein tetherin (also known as bone marrow stromal antigen 2)[95]. This clustering may affect the way in which these vesicles are internalized, favouring phagocytosis or macropinocytosis to support the engulfment of such large masses or aggregates of extracellular vesicles[151].

The specific composition of extracellular vesicles will influence their fate. The presence of amyloid precursor protein (also known as amyloid-β (A4) protein) on one exosome subtype from neuroblastoma cells specifically targets them to neurons, in contrast to a CD63-enriched exosome subtype that binds both neurons and glial cells[154]. Another example is the presence of syncytin 1 at the surface of exosomes derived from the trophoblast, which promotes their uptake[155], whereas the 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 to be 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 show largely different dynamics of interaction with membranes of microglia and astrocytes[157]. It has also been shown that filopodia drive extracellular vesicles towards sites of uptake[158]. The lipid composition of the plasma membrane of recipient cells, such as the presence of lipid rafts, 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 recipient 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 contents 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 colocalization of trophoblast-derived exosomes with early but not late endosomal structures also suggests that some internalized extracellular vesicles can escape lysosomal degradation by being re-secreted either via the early endocytic recycling pathway or by fusion of MVEs with the plasma membrane[158] (FIG. 5).

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

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). The first examples were exosomes derived from B cells and dendritic cells that were able to present antigens to T cells and induce a specific antigenic response[15,16]. Tumour-derived microvesicles were shown to carry fibronectin, which, when bound to integrin on non-transformed fibroblasts, was able to promote their anchorage-independent growth (one of the hallmarks of tumorigenesis), contributing to the acquisition of a transformed phenotype by healthy cells[163]. As another example, microvesicles generated and released by embryonic stem cells were shown to induce invasion of maternal tissue by the trophoblast, which is mediated by the interaction of laminin and fibronectin on the microvesicles with integrins along the surfaces of the trophoblast and which promotes embryo implantation[164]. A role for extracellular vesicles in the long-range transfer of morphogens to recipient cells in developing organisms was also shown[165].

Cargo delivered by extracellular vesicles can also activate various responses and processes in the recipient cell after internalization. For example, in dendritic cells, protein cargoes of exosomes derived from intestinal epithelial cells[136] or other dendritic cells[140] are processed in the endocytic compartment similarly to antigens and then used in antigen presentation, thereby contributing to immune response regulation. Extracellular vesicles could also fuse directly with the plasma membrane or endocytic membrane of recipient cells. Such processes are required to release intraluminal content in the cytoplasm of recipient cells, a key step to support the release of miRNA[62] and mRNA[166] from extracellular vesicles into recipient cells to regulate gene expression. Direct fusion of extracellular vesicles with the membrane of recipient cells also enables the exchange of transmembrane proteins and lipids. Extracellular vesicles can transport various lipid species including eicosanoids, fatty acids and cholesterol as well as lipid translocases, thereby contributing to the regulation of bioactive lipid species[167]. Under pathological situations, a good example of material transferred through extracellular vesicles is the pathological amyloid proteins, which can be either enriched at the surface of extracellular vesicles, such as PrP or amyloid-β peptide, or present intraluminally, such as TAR DNA-binding protein 43 (TDP43) and α-synuclein. Their transfer to recipient cells, requiring back fusion, has been proposed to favour transcellular spreading of amyloids[168]. Mechanisms governing fusion of extracellular vesicles with these different compartments are not yet known but could be analogous to fusogenic processes employed by viruses[169].

The ultimate and probably the most frequent fate of extracellular vesicles is their targeting to lysosomes,

Macropinocytosis
A form of regulated endocytosis that involves the nonspecific uptake of extracellular material (such as small soluble molecules, nutrients or antigens) by invagination of the plasma membrane, which is then pinched, resulting in small vesicles in the cytoplasm.

Trophoblast
Cells that form the outer layer of a blastocyst, provide nutrients to the embryo and give rise to a large part of the placenta.

Microglia
Brain glial cells that act as the first and main endogenous immune defence in the central nervous system.

Astrocytes
Star-shaped glial cells in the brain involved in nutrient supply, maintenance of extracellular ion balance and tissue repair following brain injuries.

Filopodia
Highly dynamic actin-rich cell surface protrusions used by cells to sense their external environment.

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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).

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 neurodegenerative diseases[172]. Thus, extracellular vesicles hold 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 the progression of various pathological states, for example, for assessing risk of tumour progression and metastasis or for providing early biomarkers of neurodegenerative diseases[172]. Investigations in this area have flourished, aiming to establish the groundwork for 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 less likely to trigger innate and adaptive immune responses[175]. Their use in clinical research has already demonstrated that extracellular vesicles secreted by immune cells (dendritic cells) stimulate the immune system and can therefore be exploited as antitumour vaccines[176,177]. Several clinical

Box 1 | **Methods of isolation and analysis of extracellular vesicles**

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

Several analytical methods are available and should be combined to first assess the purity, integrity and concentration of extracellular vesicles before further analysis or other experiments are performed. The most commonly used approaches for the analysis of the composition and morphology of extracellular vesicle populations include western blot analysis, nanoparticle 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 (see Further information) that centralizes extracellular vesicle studies and methodologies provides a means to standardize extracellular vesicle research to strengthen reproducibility between studies[187]. Emerging strategies are now being developed to investigate the biogenesis and 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 vesicles by live-cell imaging in cell lines and, to a limited extent, *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, microRNA, Cre recombinase)[190,191] that induce detectable signals such as modulation of expression of a reporter gene once released in recipient cells. An alternative approach using optical tweezers allows the manipulation and visualization of single vesicles at the surface of recipient cells[154]. However, so far, the field still needs *in vivo* models that allow reproducible tracking of extracellular vesicles at a single-vesicle and high spatiotemporal resolution at different stages: through their biogenesis and transit routes in secreting cells to their delivery and fate in recipient cells. Such an 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]. Still, 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.

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Suicide mRNAs/proteins
Non-mammalian enzymes or their encoding mRNAs that are able to convert an inactive drug into highly toxic metabolites, which subsequently inhibit the synthesis of nucleic acids and cause cells to initiate apoptosis.

trials involving the use of extracellular vesicle-based delivery are ongoing, for example, in the treatment of lung cancer and melanoma, which 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 therapeutic interventions where extracellular vesicles can be used (reviewed in detail elsewhere[5,179,172]). Aside from the aforementioned use of extracellular vesicles in antitumoural therapy, dendritic cells pulsed with *Toxoplasma gondii* release extracellular vesicles that confer protection against subsequent toxoplasma infection[180]. Such a strategy could be considered for fungi, bacteria, parasitic protozoa and helminths[172]. Extracellular vesicles derived from mesenchymal stem cells have been tested in animal models for treating acute kidney failure[181], myocardial infarction[182] or ischaemia[183]. Other ongoing assays are based on *in vitro* manipulation of extracellular vesicles with the loading of a particular cargo (for example, interfering RNAs, suicide mRNAs/proteins, miRNAs and 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 therapeutic 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 the bedside.

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