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1 **Shedding light on the cell biology of extracellular vesicles**

2

3 Guillaume van Niel¹, Gisela D'Angelo² and Graça Raposo²

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5 1. France Center of Psychiatry and Neurosciences, INSERM U895, Paris
6 75014, France

7

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

10

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

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13

14 **Abstract :**

15

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

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34 **[H1] Introduction**

35

36 Apart from the release of secretory vesicles by specialized cells, which carry,
37 for example, hormones or neurotransmitters, all cells are capable of secreting
38 different types of membrane vesicles, known as extracellular vesicles, and
39 this process is conserved throughout evolution from bacteria to humans and
40 plants^{1,2,3}. Secretion of extracellular vesicles has been initially described as
41 means of eliminating obsolete compounds⁴ from the cell. However, now we
42 know that extracellular vesicles are more than waste carriers, and the main
43 interest in the field is now focused on their capacity to exchange components
44 between cells — varying from nucleic acids to lipids and proteins — and to act
45 as signalling vehicles in normal cell homeostatic processes or as a
46 consequence of pathological developments^{5,6,7}.

47

48 Even though one generic term — extracellular vesicles – is currently in use to
49 refer to all these secreted membrane vesicles, they are in fact highly
50 heterogeneous (Fig. 1), which has largely hampered characterization and
51 manipulation of their properties and functions. Insights into the biogenesis of
52 secreted vesicles was provided by transmission and immuno-electron
53 microscopy, and by biochemical means⁸⁻¹⁰. Based on the current knowledge
54 of their biogenesis, extracellular vesicles can be broadly divided into two main
55 categories: exosomes and microvesicles (Fig 1a).

56

57 The term exosome (which should not be confused with the exosome complex,
58 which is involved in RNA degradation¹¹) was initially used to name vesicles of
59 an unknown origin released from a variety of cultured cells and carrying 5'-
60 nucleotidase activity¹². Subsequently, the term exosomes was adopted to
61 refer to membrane vesicles (30-100 nm in diameter) released by **reticulocytes**
62 **[G]** during differentiation⁴. In essence, exosomes are intraluminal vesicles
63 (ILVs) formed by the inward budding of endosomal membrane during
64 maturation of multivesicular endosomes (MVEs) — which are intermediates
65 within the endosomal system — and secreted upon fusion of MVEs with the
66 cell surface^{13,14} (Fig 1a-c). In the mid 1990's exosomes were reported to be
67 secreted by B lymphocytes¹⁵ and dendritic cells¹⁶ with potential functions
68 related to immune regulation, and considered for use as vehicles in anti-

69 tumoral immune responses. Exosome secretion is now largely extended to
70 many different cell types and their implications in intercellular communication
71 in normal and pathological states are now well documented⁵.

72

73 Microvesicles, formerly called “platelet dust”, were described as subcellular
74 material originating from platelets in normal plasma and serum¹⁷. Later,
75 ectocytosis, a process allowing the release of plasma membrane vesicles,
76 was described in stimulated neutrophils¹⁸. Although microvesicles were mainly
77 studied for their role in blood coagulation^{19,20}, more recently they were
78 reported to have a role in cell–cell communication in different cell types,
79 including cancer cells²¹ where they are generally called oncosomes.
80 Microvesicles range in size from 50-1000 nm in diameter, but can be even
81 larger (up to 10µm) in the case of oncosomes. They are generated by the
82 outward budding and fission of the plasma membrane and the subsequent
83 release of vesicles into the extracellular space²² (Fig 1a-c).

84

85 There is now evidence that each cell type tunes extracellular vesicle
86 biogenesis depending on its physiological state, and to release extracellular
87 vesicles with particular lipid, protein and nucleic acid compositions⁵ (Fig. 1d).
88 Because most published reports of extracellular vesicles have focused on
89 their potential functions rather their origins, it is still unclear which sub-species
90 of vesicles is responsible for any given effect. The current available protocols
91 to recover extracellular vesicles from cell culture supernatants or liquid
92 biopsies result in a heterogeneous population of vesicles of unknown origin²³.
93 Moreover, the diversity of isolated extracellular vesicle populations is further
94 expanded by the inclusion of additional structures into the pool of extracellular
95 vesicles, such as the apoptotic bodies, migrasomes, which transport
96 multivesicular cytoplasmic contents during cell migration²⁴ or arrestin domain-
97 containing protein 1-mediated microvesicles (ARMMS)²⁵, which are largely
98 uniform, ~50 nm in diameter, microvesicles that have been shown to bud
99 directly from the plasma membrane in a manner resembling the budding of
100 viruses and dependent on arrestin domain-containing protein 1 (ARRDC1)
101 and on endosomal sorting complex required for transport (ESCRT) proteins
102 (similarly to a sub-population of exosomes; see also below).

103

104

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

119

120

121 **[H1] Biogenesis of extracellular vesicles**

122

123 Exosomes and microvesicles have different modes of biogenesis (but both
124 involve membrane trafficking processes): exosomes are generated within the
125 endosomal system as ILVs and secreted during fusion of MVEs with the cell
126 surface, whereas microvesicles originate by an outward budding at the
127 plasma membrane¹⁰. This nomenclature is still questionable as extracellular
128 vesicle biogenesis pathways may differ according to the producing cell type.
129 For example, T cells generate primarily extracellular vesicles from the cell
130 surface with characteristics of exosomes, likely exploiting at the plasma
131 membrane molecular components and mechanisms that are usually
132 associated with the endosomal biogenesis of ILVs²⁸. This peculiar biogenesis
133 of exosomes from the plasma membrane might be specific to T cells, which
134 also use the endosomal machinery for HIV budding at the plasma
135 membrane²⁹.

136

137 Even though generation of microvesicles and exosomes occurs at distinct
138 sites within the cell, common intracellular mechanisms and **sorting**
139 **machineries [G]** are involved in the biogenesis of both entities. In many cases
140 these shared mechanisms hinder the possibility to distinguish among them⁵.
141 Mechanistic details of extracellular vesicle biogenesis have just started to be
142 uncovered as discussed below. First, cargoes scheduled for secretion within
143 extracellular vesicles must be targeted to the site of production, either at the
144 plasma membrane (for microvesicles) or at the limiting membrane of MVE (for
145 exosomes). Second, cargoes are enriched in the forming vesicles by a
146 stepwise mechanism of clustering and budding followed by fission and vesicle
147 release (Fig 2).

148

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

151 The nature and abundance of extracellular vesicle cargoes³⁰ (Fig 1d) is cell
152 type specific and is often influenced by the physiological or pathological state
153 of the donor cell, the stimuli that modulate their production and release, and
154 the molecular mechanisms that lead to their biogenesis³¹. Cargoes are the
155 first regulators of extracellular vesicle formation. As reported for exosomes, an
156 ectopic expression of a particular cargo such as, for example the expression
157 of the **major histocompatibility complex [G]** (MHC) class II³² promotes MVE
158 formation with a consequent release of extracellular vesicles, likely by
159 recruiting sorting machineries that will promote MVE and ILV generation^{32,33}.

160

161 Exosomal membrane cargoes reach endosomes from the Golgi apparatus or
162 are internalized from the plasma membrane before being sorted to ILVs
163 during endosome maturation³⁴ (Fig 1). Hence cargoes that are preferentially
164 recycled to the plasma membrane are likely not enriched in exosomes unless
165 their recycling is impaired, as is the case for the transferrin receptor in
166 reticulocytes³⁵. Therefore, impairment or depletion of regulators of endosomal
167 recycling and retrograde transport from endosomes to the Golgi might
168 generally affect the targeting of some cargoes to extracellular vesicles. In this
169 context, **syntenin [G]** protein, by acting both in the recycling³⁶ and in the
170 sorting of **syndecan [G]** in MVEs³⁷ for exosome biogenesis, appears as a

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

173

174 Modulation of endocytosis or recycling of cargoes to the plasma membrane
175 would also impinge on their targeting at the site of microvesicle biogenesis.
176 For example, the small GTPase ADP-ribosylation factor 6 (ARF6) was
177 identified as a regulator of selective recruitment of proteins, including β 1
178 integrin receptors, MHC class I molecules, membrane type 1-matrix
179 metalloproteinase 1 (MT1-MMP) and the vesicular soluble N-ethylmaleimide-
180 sensitive factor attachment protein receptor (v-SNARE) VAMP3 into tumour-
181 derived microvesicles^{38,39}. In addition to ARF6-regulated endosomal
182 trafficking, VAMP3 mediates the trafficking and incorporation of MT1-MMP
183 into tumor-derived microvesicles in a CD9-dependent manner. This suggests
184 that VAMP3- and ARF6-positive recycling endosomes are a site of MT1-MMP
185 recycling to the cell surface and trafficking to microvesicles. Such crosstalk
186 between recycling and microvesicle biogenesis is also illustrated by studies
187 reporting that the small GTPase Rab22a co-localizes with budding
188 microvesicles and mediates packaging and loading of cargo proteins in
189 hypoxic breast cancer cells⁴⁰.

190

191

192 ***[H3] Machineries involved in the biogenesis of exosomes.***

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

198

199 The discovery of the ESCRT machinery as a driver of membrane shaping and
200 scission was the first breakthrough into uncovering the mechanisms involved
201 in the formation of MVEs and ILVs⁴¹. ESCRT acts in a stepwise manner
202 where ESCRT-0 and ESCRT-I subunits cluster ubiquitinated transmembrane
203 cargoes on microdomains of the limiting membrane of MVEs and recruit, via
204 ESCRT-II, the ESCRT-III subcomplexes that perform budding and fission of

205 this microdomain (Fig 2). Accordingly, Hrs (ESCRT-0) appears to be required
206 for exosome formation and/or secretion by dendritic cells⁴².

207

208 The role of the ESCRT machinery in ILV biogenesis and the presence of
209 some ESCRT subunits in exosomes opened an avenue to understand and
210 modulate the formation of exosomes through manipulation of the ESCRT
211 components. A medium-throughput RNA interference screen targeting²³
212 different components of ESCRT machinery and associated proteins **highlights**
213 has revealed various roles for selected members of this family in exosomes
214 generation. Their inactivation impacts either the efficiency of secretion or the
215 composition of the secreted vesicles indicating that some ESCRT
216 components could act selectively on MVE and ILV subpopulations fated for
217 secretion as exosomes⁴³. The canonical ESCRT pathway can be intersected
218 by syntenin and ALIX (ALG-2 interacting protein X; an ESCRT accessory
219 protein; also known as PDCD6-interacting protein), which bridge cargoes and
220 the ESCRT-III subunit vacuolar protein sorting-associated protein 32 (VPS32;
221 also known as CHMP4)³⁷.

222

223 Exosomes can be also formed in an ESCRT-independent manner, which was
224 revealed by studies showing that MVEs, featuring ILVs loaded with CD63 are
225 still formed upon depletion of components of the four ESCRT complexes⁴⁴.
226 The first ESCRT-independent mechanism of exosome biogenesis was shown
227 to require generation of **ceramide [G]** by neutral type II sphingomyelinase that
228 hydrolyzes **sphingomyelin [G]** to ceramide⁴⁵. Ceramide may then allow
229 generation of membrane subdomains⁴⁶, which impose spontaneous negative
230 curvature on the membranes. Alternatively, ceramide could be metabolized to
231 sphingosine 1-phosphate to activate G_i-protein coupled sphingosine 1-
232 phosphate receptor that appears essential for cargo sorting into exosomal
233 ILVs⁴⁷ (Fig 2). In addition, proteins of the **tetraspanin family [G]**, have been
234 shown to regulate the ESCRT-independent endosomal sorting. One of these
235 proteins is CD63, which is particularly enriched on the surface of exosomes
236 and has been shown to be involved in endosomal sorting in melanocytes^{48,49},
237 in cargo (apolipoprotein E) targeting to exosomes secreted by melanoma
238 cells⁵⁰ and in the biogenesis of exosomes in fibroblasts from down syndrome

239 patients⁵¹. Tetraspanins CD81, CD82 and CD9 are also directly involved in
240 the sorting of various cargo to exosomes^{52,53}. Mechanistically, these proteins
241 form clusters and dynamic membrane platforms with other tetraspanins and
242 with different transmembrane and cytosolic proteins⁵⁴ likely acting in the
243 formation of the microdomains that will bud. Moreover, recent structural
244 analysis of the tetraspanin CD81 revealed a cone-like structure with an
245 intramembrane cavity that can accommodate cholesterol and that is likely
246 shared by other tetraspanins. Clustering of several cone-shaped tetraspanins
247 could then induce inward budding of the microdomain in which they are
248 enriched⁵⁵ (Fig 2). But tetraspanins also regulate the intracellular routing of
249 cargoes towards MVEs, such as integrins⁵⁶, which indicates that impairment
250 of their function may affect different steps of exosome generation. Thus, it
251 seems that both ESCRT-dependent and -independent mechanisms operate in
252 exosome biogenesis, and their contribution may vary depending on the
253 cargoes, which recruit them, and the cell type.

254

255 As mentioned above, sorting of transmembrane cargoes into extracellular
256 vesicles is largely dependent on endosomal sorting machineries. However,
257 additional mechanisms contribute to the targeting of selective soluble or
258 membrane associated cargoes to exosomes. For example, the sequestration
259 of cytosolic proteins into ILVs can results from co-sorting with other proteins,
260 such as chaperones heat shock protein 70 (HSP70) and heat shock cognate
261 70 (HSC70), which are found in exosomes derived from most cell types^{57,58}.
262 Membrane cargoes, such as **GlycosylPhosphatidyInostol (GPI)-anchored**
263 **proteins [G]** are present in exosomes likely because of their affinity for lipid
264 domains and **lipid rafts [G]** that could be directly involved in ILV generation
265 through their effects on biophysical properties of membranes⁵⁹. It has also
266 been proposed that some cytosolic proteins, modified by ubiquitylation⁶⁰ or
267 farnesylation⁶¹ are segregated in ILVs and in exosomes but the underlying
268 mechanisms for their enrichment in these compartments are still lacking.
269 Apart from proteins, extracellular vesicles also carry nucleic acids, including
270 RNAs (mRNAs and non-coding RNAs including micro RNAs (miRNAs)^{62,63}
271 and DNA sequences^{64,65}. Interestingly, miRNAs have been shown to be
272 differentially sorted to exosomes, depending on their sequence (presence of

273 specific motifs)⁶⁶, which indicates that incorporation of nucleic acids into
274 exosomes is regulated. However, the relative contributions of passive and
275 active loading of RNAs into extracellular vesicles remain unclear⁶⁷. The
276 mechanisms involved in targeting nucleic acids to exosomes are so far
277 elusive. Different machineries have been proposed to perform specific nucleic
278 acid sorting, including the ESCRT-II subcomplex that could act as an RNA
279 binding complex⁶⁸, the tetraspanin-enriched microdomains that could
280 sequester many RNA-binding proteins in the membrane subdomains⁶⁹ or the
281 miRNA-induced silencing complex (miRISC) and protein argonaute 2
282 (AGO2), which mediate RNA silencing processes⁷⁰. New regulators of miRNA
283 sorting into exosomes have also recently been described and include the
284 **KRAS–MEK signalling pathway [G]** acting through AGO2⁷¹, **Major Vault**
285 **protein [G]**⁷² or **Y-box protein 1 [G]**⁷³.

286

287 In sum, exosome biogenesis is certainly complex and varies depending on the
288 cargo and on the cell type and can be influenced by other signals and
289 pathological stimuli that the cell can receive. The balance of these pathways
290 leading to changes in the compositional repertoire of exosomes also changes
291 over the course of the differentiation process as reported for reticulocytes⁷⁴, or
292 during cell maturation as shown for dendritic cells⁷⁵. Accordingly, most cells
293 host subpopulations of MVEs distinguished by different lipid and protein
294 compositions and morphology^{52,76}. In this context, different sorting
295 mechanisms can act on the same endosomal compartment⁴⁹ or different
296 machineries can be used for targeting the same cargo (for example MHCII,
297 which can be targeted to MVEs by both ESCRT-dependent and independent
298 mechanisms)^{52,77}, or on different maturation products of the cargo (as is the
299 case for melanocyte protein PMEL for which its luminal domain, which is
300 generated by proteolysis, is sorted by ESCRT-independent mechanisms,
301 whereas ESCRT-dependent mechanism is involved in targeting the
302 transmembrane domain of PMEL)⁴⁹. Therefore several mechanisms could
303 concomitantly or sequentially act on forming MVEs, thereby allowing the
304 sorting of diverse cargoes at different stages of maturation of the MVE⁷⁸;
305 alternatively or concomitantly, distinct subpopulations of MVEs may exist and
306 may be targeted by different machineries^{5,49} (Fig 3). Overall, this data support

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

315

316

317

318 ***[H3] Machineries involved in the biogenesis of microvesicles.***

319 Whereas blebbing from the plasma during apoptosis has long been known to
320 produce microvesicles in the form of apoptotic bodies⁷⁹, the release of
321 microvesicles from the plasma membrane of healthy cells and the
322 mechanisms involved in this secretion have only started to emerge recently.
323 This biogenesis requires several molecular rearrangements within the plasma
324 membrane, including changes in lipid components and protein composition,
325 and in Ca²⁺ levels³¹. Ca²⁺-dependent enzymatic machineries including
326 **aminophospholipid translocases [G]** (flippases and floppases), **scramblases**
327 **[G]** and **calpain [G]** drive rearrangements in the asymmetry of membrane
328 phospholipids (exposition of phosphatidylserine from the inner leaflet to the
329 cell surface), which causes physical bending of the membrane and
330 restructuring of the underlying actin cytoskeleton, which favour membrane
331 budding and formation of microvesicles^{21,80} (Fig 2). A genetic defect in the
332 activity of the lipid scramblase suppresses the exposure of phosphatidylserine
333 on blood platelets, and the production of procoagulant-containing
334 microvesicles⁸⁰. However, even when the membrane lipid asymmetry is
335 maintained, microvesicle biogenesis might proceed^{81,82}. These observations
336 suggest that other lipids, and the domains they form, contribute to
337 microvesicle biogenesis. One important lipid component is cholesterol, which
338 is abundant in microvesicles and pharmacological depletion of which impairs
339 their generation in activated neutrophils⁸³.

340

341 In addition to lipids, cytoskeletal elements and their regulators are certainly
342 required for microvesicle biogenesis. The activity of **Rho family of small**
343 **GTPases [G]** and of the Rho-associated protein kinase (ROCK), which are
344 important regulators of actin dynamics, induce microvesicle biogenesis in
345 different populations of tumor cells⁸⁴. As another example, in the enterocyte
346 **brush border [G]**, myosin 1a distributed along the microvillar tips exerts plus
347 end-directed force on the apical membrane, leading to the formation and
348 release of gut microvesicles⁸⁵.

349

350 The biogenesis of tumor-derived microvesicles (oncosomes) is also tightly
351 associated with metabolic changes, the so-called **Warburg effect [G]**⁸⁶. In
352 breast cancer cells, elevated glutaminase activity is dependent on Rho
353 GTPases⁸⁷, and inhibition of its activity blocks microvesicle biogenesis. This
354 suggest that formation and loading of microvesicles is linked to their metabolic
355 capability and to the Rho-GTPase signalling pathway, even beyond its role in
356 actomyosin regulation.

357

358 As for cargo targeting to exosomes, lipids and other membrane associated
359 cargoes are localized to sites of microvesicle budding through their affinity for
360 lipid rafts or as is the case for oligomeric cytoplasmic proteins, by their
361 anchoring to plasma membrane lipids^{88,89} — two mechanisms that are
362 strikingly analogous to the budding of HIV and other retroviruses. Cytosolic
363 components fated for secretion into microvesicles require their binding to the
364 inner leaflet of the plasma membrane. This association is dependent on their
365 respective plasma membrane anchors (palmitoylation, prenylation,
366 myristoylation) and the establishment of high-order complexes, that
367 concentrates them to the small membrane domains from which forming
368 microvesicles will bud^{88,89}. It is still unclear how nucleic acids, which are
369 generally found in microvesicles, are targeted to cell surface. One possible
370 mechanism revealed from studies of cancer cells suggests the involvement of
371 conserved **zipcode RNA sequence motifs [G]** in the 3' untranslated regions in
372 mRNA targeting into microvesicles⁹⁰, but the details of this process remain to
373 be discovered.

374

375 **[H1] The release of extracellular vesicles**

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

399

400 **[H3] Avoiding MVE degradation.**

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

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

414

415 Some insights into how the balance between targeting MVEs for secretion
416 and degradation is established have recently emerged. A first level of
417 regulation of this balance is likely imposed by the sorting machineries at
418 MVEs. While the different components of ESCRT machinery have various
419 effect on exosomes secretion²³ and generally associated with degradative
420 MVE, the syndecan–syntenin–ALIX pathway seems to be restricted so far to
421 exosome secretion³⁷. On the same line, MHCII is targeted to MVEs fated for
422 lysosomal degradation through ubiquitination (likely recruiting ESCRT
423 machinery) while ubiquitin- (and likely ESCRT-) independent mechanisms
424 target MHC II to MVEs fated for secretion^{52,77}. The mechanisms underlying
425 this balance are still unclear but involve components of various sorting
426 machineries such as ESCRT-I component tumour susceptibility gene 101
427 protein (TSG101), whose **ISGylation [G]** favours lysosomal degradation (and
428 thereby impairment of exosome secretion)⁹⁴, or the tetraspanin 6⁹⁷,
429 overexpression of which slows down lysosomal degradation likely by
430 recruiting sorting machinery that involves the syntenin pathway. These
431 findings are in accordance with the involvement of ESCRT-independent
432 machineries in the generation of MVEs fated for exosome secretion but not for
433 lysosome degradation^{49,52,98}.

434

435 A similar balance exists between exosome secretion and macroautophagy —
436 the process that drives degradation of superfluous or damaged cellular
437 components in the lysosome to maintain cellular homeostasis and that
438 promotes energy conservation under stress. More specifically, the fusion of
439 MVEs with autophagosome would promote their degradation and prevent
440 exosome secretion⁹⁹ (Fig 4). In this context, it has been shown that the prion
441 protein (PrP) can promote exosome secretion by inhibiting autophagosome
442 formation and it does so by interacting with **caveolin [G]** and modulating its

443 inhibitory effect on autophagosome formation¹⁰⁰. Of interest, chemical
444 inhibition of autophagy increases the recovery of autophagosome-associated
445 proteins in the isolated exosomal pellet but not of exosome-enriched
446 proteins¹⁰¹. This suggests that the capacity of MVEs to secrete exosomes is
447 counter-balanced by their fusion with the autophagosome. Yet,
448 autophagosomes and MVEs can both secrete their content but the molecular
449 mechanisms regulating these secretory pathways are likely distinct.

450

451 ***[H3] Transport of MVEs.***

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

457

458 In general, intracellular transport involves the association of organelles with
459 the cell cytoskeleton (actin, microtubules), associated molecular motors
460 (dynein, kinesins, myosins) and molecular switches (Small GTPases)^{102,103}.

461 Exosome secretion is provided by the oriented secretion of these vesicles
462 towards the immunological synapse between antigen-presenting cells and T
463 cells during antigen presentation^{52,104}. This implies that at least in the context
464 of immunological synapse MVEs follow the network of microtubules oriented
465 by the microtubule organizing centre (typically the centrosome)⁹¹ (Fig 4). The
466 molecular motors involved in this process remain to be determined but
467 certainly counterbalance those that regulate transport of MVEs towards
468 lysosomes. Targeting to lysosomes occurs by a retrograde transport on
469 microtubules (towards microtubule minus ends), and Rab-GTPase Rab7 and
470 its associated proteins promote the recruitment of the retrograde molecular
471 motor dynein that targets MVE to lysosomes¹⁰⁵. Interestingly, Rab7 is also
472 mandatory for the release of exosomes³⁷. These dual effect on exosome
473 secretion seems to rely on the ubiquitylation status of Rab7, which has been
474 shown to promote the recruitment of the machinery involved in lysosomal
475 targeting of MVEs at the expense of exosome secretion¹⁰⁶. Curiously, in
476 endosomes the recruitment of Rab7 leading to lysosomal targeting is

477 stimulated by cholesterol at their limiting membrane, whereas MVE-containing
478 ILVs enriched in cholesterol have been shown to undergo preferential
479 secretion as exosomes¹⁰⁷. Thus, dynamic changes in the composition of the
480 limiting membrane of MVEs, through incorporation of specific lipids and
481 proteins into ILVs, would likely regulate the fate of MVEs towards degradation
482 or secretion.

483

484 Rab27a and Rab27b³² and their respective effectors, synaptotagmin-like
485 protein 4 and exophilin 5, are also essential for exosome secretion. Rab27b
486 regulates the motility of MVEs towards the plasma membrane, and both
487 Rab27 isoforms act on the step following MVE transport, that is the docking at
488 the plasma membrane to promote fusion, thereby increasing exosome
489 secretion. The role of Rab27a in MVE docking involves rearrangement of sub-
490 membrane actin cytoskeleton¹⁰⁸, a step that is common to all mechanisms
491 involving vesicular secretion. Rab27 also controls secretion of secretory
492 lysosomes so called lysosome related organelles¹⁰⁹, which suggests that
493 MVEs capable of exosome secretion may be considered as a specialized
494 compartment rather than a simple MVE subtype. Of note, Rab27 isoforms are
495 not constitutively expressed in all cell types, which implies that each cell type
496 may adapt its own secretory machineries for exosome secretion. This is
497 illustrated by reported involvement of additional Rabs and their effectors, such
498 as Rab11 and Rab35 effector^{110,111} in the direct regulation or the potential
499 priming of MVE secretion.

500

501 ***[H3] Fusion of MVEs with the plasma membrane.***

502 The final step of exosome secretion requires the fusion of MVEs with the
503 plasma membrane to release ILVs as exosomes (Fig 4), a process likely
504 mediated by **SNARE proteins [G]** and **synaptotagmin family [G]** members¹¹².
505 A SNARE complex known to be implicated in the exocytosis of conventional
506 lysosomes consists of VAMP7 on the lysosomes, syntaxin 7 on the plasma
507 membrane and the lysosomal regulatory protein synaptotagmin 7¹¹³. This
508 complex is involved in exosome secretion in some cells (human leukemia
509 K562 cell line)¹¹⁴ but not in others (MDCK cells)¹¹⁵. The process of exosome
510 secretion has been demonstrated in several cell types to be regulated by Ca²⁺

511 ¹¹⁶⁻¹¹⁸, which may have a role in the activation of the SNARE complexes. The
512 implication of SNAP23 — a SNARE shown to regulate lysosome-related
513 organelles secretion in mastocytes¹¹⁹ — also in exosome secretion¹²⁰,
514 strengthens the notion that MVEs are indeed specialized secretory organelles.
515 Additional SNARE proteins involved in exosome secretion such as YKT6 ¹²¹ in
516 *Drosophila*, SYX-5 in *C. elegans* ¹²² and syntaxin 1a ¹²³ in mammals reflect
517 again the diversity of regulators that could be involved in exosome secretion,
518 most likely depending on the organism, the cell type or the MVE subtypes. It
519 should be noted that most of the studies on the intracellular regulators of
520 exosome release came from analysis of exosomal pellets isolated from
521 supernatants from cell cultures treated with inhibitors or interfering RNAs
522 against potential targets, ignoring the complexity of intracellular pathways that
523 might be affected in the producing cells by these perturbations. Moreover, the
524 quantity of extracellular vesicles recovered in the supernatant does not take
525 into account the fraction of vesicles that remains tethered (not fully released)
526 at the plasma membrane of the producing cells⁹⁵ or the fraction of
527 extracellular vesicles that can be recaptured by the same cell¹²⁴. A better
528 understanding of this step certainly requires the development of new tools and
529 techniques to follow docking and fusion of MVEs with the plasma membrane.

530

531 **[H3] Release of microvesicles.**

532 The release of microvesicles requires their fission from the plasma
533 membrane, a mechanism that is dependent on the interaction of actin and
534 myosin with a subsequent ATP-dependent contraction^{85,125}. As such, the
535 activation of small GTP binding proteins including ARF6 and ARF1 leads to
536 the phosphorylation of the myosin light chain (MLC) and actomyosin
537 contraction, which allows the vesicles to bud off from the membranes of
538 cancer cells^{39 126 127}. In HeLa cells another regulator of actin dynamics, Cdc42
539 has been shown to be involved, but the underlying mechanism is still not
540 known⁸⁴. Interestingly, TSG101 and VPS4-ATPase, mostly involved in
541 exosomes generation as part of the ESCRT machinery, were reported to
542 participate in the scission and release of ARMMs (subtype of microvesicles
543 containing ARRDC1)²⁵. Shedding of ESCRT-dependent microvesicles was
544 also reported in *C. elegans* embryos upon loss of the conserved flippase P4-

545 ATPase, TAT-5, which leads to the cytosolic exposure of
546 phosphatidylethanolamine, an aminophospholipid asymmetrically enriched in
547 the inner leaflet of the membrane bilayer¹²⁸. This scenario mirrors the
548 exposure of phosphatidylserine by lipid translocation, which as discussed
549 above, can promote membrane bending and microvesicle budding. (Fig 2)

550

551 The involvement of cell signalling pathways in microvesicle release is strongly
552 supported by reports showing that removal of serum, and therefore growth
553 factors acting on their respective receptors and downstream effectors,
554 prevents microvesicle release¹²⁹. What is known is that a strong microvesicle
555 release is induced by increased concentration of Ca^{2+} , which by activating
556 scramblase and calpain leads to a loss of membrane phospholipid asymmetry
557 and the reorganization of the cytoskeleton (see above) or by the activation of
558 **protein kinase C [G]** by phorbol esters¹³⁰. Release of microvesicles has also
559 been shown to depend on ATP-mediated activation of **P2x₇ receptors [G]**,
560 which leads to rearrangements of the cell membrane^{131,132}. Mechanistically,
561 this process is associated with the translocation of the acidic
562 sphingomyelinase to the plasma membrane where it generates ceramide,
563 thereby promoting membrane bending and microvesicle shedding¹³³. The
564 involvement of acidic rather than neutral sphingomyelinase in microvesicle
565 release suggests that different members of the sphingomyelinase family
566 control the biogenesis of exosomes⁴⁵ (see above) and the release of
567 microvesicles, but in both cases, these mechanisms would support ESCRT -
568 independent vesicle release.

569

570 **[H1] Targeting to recipient cells**

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

579 cargoes are not fully unravelled. These processes are complex and depend
580 on the origin of extracellular vesicles and on the identity and origin of the
581 recipient cells, as well as seem to be linked to the downstream effects and
582 processes instigated by these vesicles¹³⁴. Current studies have been mostly
583 focused on investigating membrane interaction and intercellular fate of pools
584 of exosomes, but despite different content and size, the principles of uptake
585 and general intercellular trafficking of different sub-populations of extracellular
586 vesicle are likely to be shared.

587

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

589 Target cell specificity is likely to be determined by specific interactions
590 between proteins enriched at the surface of extracellular vesicles and
591 receptors at the plasma membrane of the recipient cells, as for example, in
592 **follicular dendritic cells [G]**¹³⁵, intestinal epithelial cell¹³⁶, dendritic cells¹³⁷, or
593 neurons¹³⁸, and also in liver, lungs or lymph nodes^{136,139}. Of note, the
594 recipient cells can also be the producing cell itself, leading to autocrine
595 responses¹²⁴.

596

597 Several mediators **[Au:OK?]** OK of these interactions are known and include
598 tetraspanins, integrins, lipids, **lectin [G]** or heparan sulfate **proteoglycans [G]**,
599 and extracellular matrix (ECM) components (Fig 5 inset). The cellular and
600 molecular basis for the specific targeting to acceptor cells is still unclear,
601 although some data is available. For example, integrins on extracellular
602 vesicles can interact with adhesion molecules such as **intercellular adhesion**
603 **molecules [G]** (ICAMs)¹⁴⁰ at the surface of recipient cells. In addition, the
604 interaction of integrins with extracellular matrix proteins, mostly fibronectin
605 and laminin, has been shown to have important roles in exosome^{141,142} and
606 microvesicle¹⁴³ binding to recipient cells. In this context, the ECM can act as a
607 “zipper” between integrins present on extracellular vesicles and target cells. *In*
608 *vivo*, integrin heterodimers may drive extracellular vesicles towards specific
609 target organs¹³⁹. One example includes exosomes released by cancer cells,
610 which can be targeted to specific organs such as lung and liver, to promote
611 premetastatic niche formation in a manner dependent on their integrin

612 composition¹³⁹. Exosomal tetraspanins could also regulate cell targeting. They
613 have been shown to interact with integrins¹⁴⁴ 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 exosome¹⁴⁷ and
620 microvesicle¹⁴⁸ 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 phagocytosis¹⁵¹⁻¹⁵³ as well as through endocytosis via caveolae and lipid
631 rafts¹⁵⁷⁻¹⁵⁹ (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 tetherin⁹⁵. 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 vesicles¹⁵¹.

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 cells¹⁵⁴.
642 Another example is the presence of syncytin at the surface of exosomes
643 derived from the **trophoblast [G]** that promotes their uptake¹⁵⁵, whereas the
644 presence of a “don’t eat me” signal , such as CD47, at the surface has been

645 shown to have a strong inhibitory effect on vesicle phagocytosis by
646 monocytes¹⁵⁶.

647

648 The fate of extracellular vesicles is also likely related to the presence of
649 specific structures at the plasma membrane of the target cell. As an illustrative
650 example, it has been shown that microvesicles derived from **microglia [G]**
651 show largely different dynamics of interaction with membranes of microglia
652 and **astrocytes [G]**¹⁵⁷. It has also been shown that **filopodia [G]** drive
653 extracellular vesicles toward sites of uptake¹⁵⁸. The lipid composition of the
654 plasma membrane of recipient cells such as the presence of lipid raft also
655 contributes to extracellular vesicle internalization as disruption of lipid rafts by
656 cholesterol depletion reduces uptake of extracellular vesicles¹⁵⁹.

657

658 Following interaction with the plasma membrane of recipients cells¹⁵⁷ and after
659 uptake by different mechanisms, extracellular vesicles follow the endocytic
660 pathway and reach MVEs, which in most cases, are targeted to the
661 lysosome^{160,161}. In some cases the internalized vesicles may escape digestion
662 by back fusion with the limiting membrane of the MVE, thereby releasing their
663 content into the cytoplasm of the recipient cell¹⁶², a process that is still poorly
664 understood but of prime importance to release intraluminal nucleic acid
665 structures (Fig 5). The restricted co-localization of trophoblast-derived
666 exosomes with early but not late endosomal structures also suggests that
667 some internalized extracellular vesicles could escape lysosomal degradation
668 by being re-secreted either via the early endocytic recycling pathway or by
669 fusion of MVEs with the plasma membrane (Fig 5)¹⁵⁵.

670

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

674

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

676 Once docked at the plasma membrane, extracellular vesicles can elicit
677 functional responses by binding to and activating receptors expressed on the
678 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 cells¹⁶³. 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 implantation¹⁶⁴. The role of extracellular vesicles in
689 the long-range transfer of morphogens to recipient cells in developing
690 organisms was also shown¹⁶⁵.

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 cells¹³⁶ or other dendritic cells¹⁴⁰ 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 miRNA⁶² and mRNA¹⁶⁶ 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 species¹⁶⁷. 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 intraluminally such
711 as TDP43 and alpha-synuclein. Their transfer to recipient cells, requiring back
712 fusion, has been proposed to favour transcellular spreading of amyloids¹⁶⁸.

713 Mechanisms governing fusion of extracellular vesicles with these different
714 compartments are not known yet, but could be analogous to fusogenic
715 processes employed by viruses¹⁶⁹.

716

717 The ultimate and likely most frequent fate of extracellular vesicles is their
718 targeting to lysosomes, which leads to the degradation of proteins and lipids
719 carried by extracellular vesicles. Of importance this degradative pathway
720 would provide a relevant source of metabolites to the recipient cells¹⁷⁰ (Fig 5).

721

722 **[H1] Conclusions and perspectives**

723 Much progress has been made in recent years in understanding the basic
724 biology of extracellular vesicles, but further investigations are required to fully
725 resolve the functional capabilities of these vesicles. Extracellular vesicles are
726 involved in several physiological contexts and pathological states, including
727 blood coagulation, inflammation, stem cell expansion, neuronal
728 communication and tumorigenesis among others⁶. In this context, extracellular
729 vesicles have been shown to carry, for example, tumour-associated
730 molecules in case of cancer and premetastatic niche establishment^{139 171}, or
731 particular components associated with neurodegenerative diseases¹⁷². Thus,
732 extracellular vesicles hold a great potential for clinical application.

733

734 Regulatory pathways involved in biogenesis and secretion of extracellular
735 vesicles, when well defined, could be used to manipulate extracellular vesicle
736 generation in pathological states, such as tumorigenesis, where the
737 involvement of extracellular vesicles in pathology has been particularly well
738 documented¹⁶³. Nevertheless, it should be noted that manipulation of
739 machineries involved in the biogenesis, transport or targeting of extracellular
740 vesicles for therapeutic benefit should be approached with caution, owing to
741 potential secondary effects of such manipulations on healthy tissues¹⁷³.

742

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

747 neurodegenerative diseases¹⁷². Investigations in this area have flourished,
748 aiming to put on solid ground the use of extracellular vesicles as biomarkers
749 in a variety of diseases. Developing techniques to enrich for disease-
750 associated (for example, tumour-derived) extracellular vesicles to define their
751 selective cargo can improve the sensitivity of such biomarkers¹⁷⁴. Whether
752 these “membrane biomarkers” correspond to endosomal-derived exosomes or
753 membrane-derived microvesicles is so far unclear although potentially
754 informative. Future studies and optimized isolation procedures (Box 1) will
755 shed light on the nature of the different extracellular vesicle subpopulations
756 that could be associated with distinct pathological states and stages of
757 progression of a given disease.

758

759 Another emerging application is the use of microvesicles and exosomes as
760 vectors for the delivery of defined compounds or more generally for
761 modulation of cell functions in an *in vivo* context. Extracellular vesicles are
762 biocompatible, can be immunologically inert, and can, if necessary, be
763 patient-derived and therefore with lower propensity to trigger innate and
764 adaptive immune responses¹⁷⁵. Their use in clinical research have already
765 demonstrated that extracellular vesicles secreted by immune cells (dendritic
766 cells) stimulate the immune system and can therefore be exploited as anti-
767 tumor vaccines^{176,177}. Several clinical trials involving the use of extracellular
768 vesicle-based delivery are ongoing, for example for the treatment of lung
769 cancer and melanoma, that may become part of an immunotherapy approach
770 that has great potential for patients with advanced cancers¹⁷⁸. Given that
771 extracellular vesicles (in particular exosomes) can be either
772 immunostimulatory or tolerogenic (immunologically inert), there are several
773 examples of possible therapeutical interventions where extracellular vesicles
774 can be used (reviewed in detail elsewhere^{5,179,172}). Beside the aforementioned
775 use of extracellular vesicles in antitumoral therapy, dendritic cells pulsed with
776 *Toxoplasma gondii* release extracellular vesicles that confer protection
777 against subsequent toxoplasma infection¹⁸⁰. Such strategy could be
778 considered for fungi, bacteria, parasitic protozoa and helminths¹⁷².
779 Mesenchymal stem cells-derived extracellular vesicles are now tested in
780 animal models to treat acute kidney failure¹⁸¹, myocardial infarction¹⁸² or

781 ischemia¹⁸³. Other undergoing assays are based on *in vitro* manipulation of
782 extracellular vesicles with the loading of a particular cargo (for example
783 interfering RNAs; suicide mRNA/protein [G], miRNAs, drugs) to then deliver it
784 to the target cell as a drug or for bioengineering purposes^{184,185}. Modulating
785 the specificity of targeting extracellular vesicles to recipient cells will be key for
786 their use as high precision vehicles, and such approaches have already been
787 tested to optimize the delivery of siRNAs to the brain¹⁸⁴.

788

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

796

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1350

1351 **Display items**

1352

1353 **Box 1**1354 Methods of isolation and analysis of extracellular vesicles.

1355

1356 The release of extracellular vesicles in the extracellular space allows their
1357 recovery from cell culture supernatants and liquid biopsies. Isolation
1358 procedures include differential ultracentrifugation, flotation on density
1359 gradient, separation by size exclusion chromatography, poly(ethylene glycol)
1360 (PEG) precipitation, immunoprecipitation and commercial kits that are partly
1361 based on these methods. These steps allow to concentrate and to separate
1362 extracellular vesicles from protein aggregates, lipoparticles, viruses, and cell
1363 debris with different rates of success. Combination of different isolation
1364 procedures is encouraged to notably separate subpopulations of vesicles
1365 based on their size, density or composition.

1366

1367 Several analytical methods are available and should be combined to first
1368 assess purity, integrity and concentration of extracellular vesicles before
1369 further analysis or other experiments. The most commonly used approaches
1370 for the analysis of the composition and morphology of extracellular vesicle
1371 populations include western-blot analysis, nano-particle tracking, transmission
1372 electron microscopy and flow cytometry and can be completed by proteomics,
1373 lipidomics and RNA and/or DNA sequencing^{67,186}. Functional analysis of
1374 extracellular vesicles depends on the question to be addressed. It should
1375 always be performed after assessment of the purity of the extracellular vesicle
1376 pellet, as soluble proteins such as cytokines, protein complexes and
1377 aggregates or lipoparticles are a major source of false positive results in
1378 functional assays.

1379

1380 A crowdsourcing knowledgebase (<http://evtrack.org>) that centralizes
1381 extracellular vesicle studies and methodologies provide a means to
1382 standardize extracellular vesicle research to strengthen reproducibility
1383 between studies¹⁸⁷. Emerging strategies are now developed to investigate
1384 biogenesis, uptake of extracellular vesicles and the transfer of material to
1385 recipient cells *in vitro* and *in vivo*. These strategies are mainly based on the

1386 labelling of isolated extracellular vesicles with fluorescent dyes or expression
1387 of fluorescent reporters that are targeted to these vesicles. Such labelling has
1388 some limitations¹⁸⁸ but allows the tracking of extracellular vesicle by live cell
1389 imaging in cell lines and, to limited extent, also *in vivo*¹⁸⁹. The main limitation
1390 being the size of the vesicles, super-resolution microscopy is one option to
1391 assess vesicle budding at the plasma membrane or in multivesicular
1392 endosomes, and to track their fate in recipient cells. A second approach is
1393 based on the loading of extracellular vesicles with molecules (mRNA, micro
1394 RNA, Cre recombinase)^{190,191} which induce detectable signals such as
1395 modulation of expression of a reporter gene once released in recipient cells.
1396 Alternative approach using optical tweezers allows the manipulation and the
1397 visualization of single vesicle at the surface of recipient cells¹⁵⁷. But, so far,
1398 the field is still in demand of *in vivo* models that would allow reproducible
1399 tracking of extracellular vesicles at a single vesicle and high spatio-temporal
1400 resolution at different stages: through their biogenesis and transit routes in
1401 secreting cells to their delivery and fate in the recipient cells. Such approach
1402 would overcome the actual limitations linked to, for example, the biased
1403 recovery of extracellular vesicles from the supernatant (resulting from
1404 processes such as vesicle tethering to the membrane of the producing cell or
1405 their re-uptake)^{95,124}. But as in any novel field of research, pre-analytical and
1406 analytical methods for studying extracellular vesicles are bound to evolve and
1407 to be better standardized to render the increasing numbers of publications in
1408 this field comparable.

1409

1410 **Figure Legends**

1411 Figure 1: Main features of extracellular vesicles.

1412 (a) Extracellular vesicles comprise a heterogeneous population of membrane
1413 vesicles of various origins. Their size may vary (typically between 50 to 500
1414 nm but they can be even larger measuring 1-10 μm). Through the last two
1415 decades, extracellular vesicle have been denominated according to their
1416 origin (cell type), size, morphology and cargo content but can now be
1417 classified into two distinct classes: exosomes and microvesicles. (b)
1418 Extracellular vesicles are formed either by budding of the plasma membrane,

1419 and are referred to as microvesicles or as intraluminal vesicles (ILVs) within
1420 the lumen of multivesicular endosomes (MVEs). MVEs fuse with the plasma
1421 membrane to release ILVs that are then called exosomes. (c) Processing of
1422 extracellular vesicles for observation by conventional transmission electron
1423 microscopy (TEM) causes their shrinking leading to an artefactual cup-shaped
1424 morphology (top panel). But when observed in close to native state by cryo
1425 electron microscopy (cryo-EM) they appear as round shaped double-leaflet-
1426 membrane enclosed structures (bottom panel). (d) Study of extracellular
1427 vesicle composition revealed that they can carry versatile cargoes, including
1428 proteins, lipid and nucleic acids and this content can largely vary between
1429 cells and conditions. The particular composition will directly affect the fate and
1430 function of extracellular vesicles, strengthening the importance of selective
1431 cargo sorting mechanisms. Of note, according to the cell type extracellular
1432 vesicles will display a set of cell type specific proteins that account for their
1433 specific fates and functions. Despite a different mode of biogenesis,
1434 exosomes and microvesicles display similar appearance, overlapping size
1435 and often common composition that make it difficult to ascertain their origin
1436 once isolated from the extracellular medium or from biological fluids. ARMMS,
1437 arrestin domain-containing protein 1-mediated microvesicles; GAPDH,
1438 glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; ICAM,
1439 intercellular adhesion molecule; TSPAN: tetraspanin; LBPA: lyso-bis-
1440 phosphatidyl acid, ICAM: InterCellular Adhesion Molecule, MHC major
1441 histocompatibility Complex, APP: amyloid precursor protein, PMEL:
1442 premelanosomal protein, TCR: T cell receptor, HSPG heparan sulfate
1443 proteoglycan, CXCR4: C-X-C chemokine receptor type 4, PrP, Prion Protein,
1444 TfR: transferrin receptor, ALIX: ALG-2 interacting protein X, Tsg101 Tumor
1445 suppressing gene 101, vps: vacuolar protein sorting, LFA-1: Lymphocyte
1446 function associated antigen 1., TDP43: TAR binding protein 43, GAPDH:
1447 glyceraldehyde-3-phosphate dehydrogenase, ERK: Extracellular signal-
1448 regulated kinases, PLD: Phospholipase D. Images in part c are the courtesy
1449 of Roberta Palmulli (G. Raposo' laboratory, URM144, Institut Curie) for
1450 conventional TEM and of Daniel Levy (UMR168, Institut Curie).

1451

1452 Figure 2: Biogenesis of extracellular vesicles

1453 Several sorting machineries are involved in the different steps required for
1454 exosomes and microvesicles generation. First, lipids and membrane-
1455 associated proteins are clustered in discrete membrane microdomains of the
1456 plasma membrane for microvesicles (top) and the limiting membrane of the
1457 multivesicular endosome (MVE) for exosomes (bottom) **(step 1)**. Such
1458 microdomains certainly also participate in the recruitment of soluble
1459 components fated for sorting in extracellular vesicles such as cytosolic
1460 proteins and RNA species **(step 2)**. Altogether formation of these clustered
1461 microdomains together with additional machineries promote membrane
1462 budding followed by a fission process either at the plasma membrane towards
1463 the extracellular medium or at the limiting membrane of the MVE towards the
1464 lumen of MVE **(step 3)**. Transmembrane proteins sorted on exosomes and
1465 microvesicles keep the same topology as at the plasma membrane.
1466 Mechanisms of exosome biogenesis are relatively well understood and
1467 importantly involve subunits of endosomal sorting complex required for
1468 transport (ESCRT), although to a different degree — ESCRT-III is required for
1469 the scission of the intraluminal vesicles (ILVs) into the MVE lumen, but
1470 membrane budding can occur by either ESCRT-dependent or ESCRT-
1471 independent mechanisms. The mechanisms involved in the biogenesis of
1472 microvesicles is still expanding. Notably, the molecular machineries that act at
1473 the different steps of extracellular vesicle biogenesis are at least partly
1474 common to exosomes and microvesicles (prominently including ESCRT
1475 proteins but also generation of ceramide through the action of different types
1476 of sphingomyelinase). One exception is the flipping of specific lipid species
1477 between the leaflets of the budding membrane that has been uniquely
1478 reported so far to microvesicle budding. Thus, it is difficult to ascertain the
1479 origin of the produced extracellular vesicle by simply impairing the function of
1480 a given mechanism involved in biogenesis of these vesicles. ARF6, ADP-
1481 ribosylation factor 6.

1482

1483 Figure 3: Origin of exosome diversity in relation to sorting machineries.

1484 The diversity of extracellular vesicle sub-populations with distinct composition
1485 and function that are generated by a given cell type is often attributed to the

1486 production of distinct sets of exosomes and microvesicles^{23,192}. This diversity
1487 is well-exemplified by the secretion of different exosomes sub-populations —
1488 with distinct morphology and composition — from apical and basolateral sides
1489 of polarized cells such as intestinal epithelial cells^{193 194 195}. Such exosome
1490 sub-populations are likely originating from sub-populations of MVEs. The
1491 distinct composition of these subpopulations reflects on the presence of
1492 multiple sorting machineries that act on the MVE compartment. It remains to
1493 be determined whether the different sorting machineries act on distinct sub-
1494 populations of MVEs (see part on right) or concomitantly in single MVEs to
1495 generate distinct sub-populations of intraluminal vesicles (ILVs) (see part on
1496 the left). The reality is most likely a mix of these two possibilities, with distinct
1497 contribution depending on the cell type. Recruitment of a given sorting
1498 machinery can depend on the maturation stage of the producing cells^{52 74}, on
1499 the post-translational modification of the cargo (for example: proteolytic
1500 processing, ubiquitylation)^{49,193} or potentially on the stage of maturation of
1501 MVEs. The type of sorting machinery recruited to MVEs will also specify the
1502 fate of MVEs between exosome secretion and lysosomal degradation.
1503 ESCRT, endosomal sorting complex required for transport.

1504

1505 Figure 4: Interdependency of intracellular trafficking routes in the generation
1506 of extracellular vesicles

1507 The generation of exosomes and microvesicles requires a tuned regulation of
1508 multiple intracellular trafficking steps (blue arrows for exosomes, green arrows
1509 for microvesicles) that influence the targeting of cargoes to the site of
1510 extracellular vesicle biogenesis as well as for exosomes, the fate of the
1511 multivesicular endosome (MVE) from which these vesicles originate. Cargoes
1512 targeted to MVEs originate from endocytosis at the plasma membrane or are
1513 directly targeted to MVEs or to early sorting endosomes via the biosynthetic
1514 pathway (from the trans-Golgi Network (TGN)). Retrograde transport towards
1515 the TGN or recycling back to the plasma membrane will divert cargoes from
1516 their targeting to MVE (dashed arrows) and therefore their incorporation into
1517 ILVs. These sorting processes are regulated by various Rab GTPases. Once
1518 matured, MVEs that are not targeted to lysosomes or autophagosomes for
1519 degradation are transported to the plasma membrane on microtubules. At this

1520 step, docking and fusion are the two final processes required for exosome
1521 release. Rabs, actin and soluble N-ethylmaleimide-sensitive factor attachment
1522 protein receptor (SNARE) proteins are involved in these steps of exosome
1523 release. In the case of microvesicle biogenesis, endocytic uptake (dashed
1524 arrow) and recycling will respectively decrease and increase the targeting of
1525 membrane (and membrane bound) cargoes to microvesicles. Of note, as the
1526 release of exosomes requires tightly regulated steps of transport, tethering
1527 and fusion of MVE to plasma membrane (apart from cargo sorting) this could
1528 account for the time difference between the generation and release of both
1529 types of extracellular vesicles. OK * denotes proteins of *Caenorhabditis*
1530 *elegans*.

1531

1532

1533 Figure 5: Fate of extracellular vesicles in recipient cells.

1534 In recipient cell (which can be the producing cell itself) exogenous
1535 extracellular vesicles will bind to the cell surface (see inset) and can follow
1536 various fates. Depending on the cell type they can remain bound to the
1537 surface (for example to integrins) and can initiate intracellular signalling
1538 pathways (for example antigen presentation). Extracellular vesicles may also
1539 be internalized by multiple routes. Internalization will target exogenous
1540 extracellular vesicles into the canonical endosomal pathway, whereby they
1541 reach multivesicular endosomes (MVEs) where the uptaken vesicles will likely
1542 mix with endogenous intraluminal vesicles (ILVs). Fusion of MVEs with the
1543 lysosome will lead to the degradation of extracellular vesicles and the
1544 recycling of their content to fuel recipient cell metabolism. Extracellular
1545 vesicles either docked at the plasma membrane or at the limiting membrane
1546 of MVEs can release their intraluminal content into the cytoplasm of the
1547 recipient cell by fusion, a process which is so far poorly understood but of
1548 major importance for delivery of intraluminal cargoes such as miRNA. Of note,
1549 there is no evidence so far to exclude potential recycling to the plasma
1550 membrane of endocytosed vesicles (dashed arrows). ECM, extracellular
1551 matrix; ICAM, intercellular adhesion molecules; TIM4, T-cell immunoglobulin
1552 mucin receptor 4.

1553

1554

1555 **Glossary**

1556 **reticulocytes**: precursors of red blood cells (erythrocytes)

1557

1558 **sorting machineries**: protein complexes mediating cargo sorting in
1559 endosomes

1560

1561 **major histocompatibility complex**: a group of genes that code for cell-
1562 surface glycoproteins that help the immune system to determine self and
1563 nonself

1564

1565 **syntenin**: an intracellular adaptor protein linking syndecan-mediated
1566 signalling to the cytoskeleton

1567

1568 **syndecan**: a single transmembrane domain heparan sulfate proteoglycan that
1569 binds a large variety of ligands, such as growth factors and fibronectin among
1570 others

1571

1572 **ceramide**: a lipid molecule composed of sphingosine and a fatty acid linked
1573 through an amide bond; in fact, many chemically diverse ceramides have
1574 been described, showing that ceramide is not a single molecular species but
1575 rather a family of related molecules.

1576

1577 **sphingomyelin**: a type of sphingolipid found in animal cell membranes.

1578

1579 **tetraspanin family**: family of proteins with four transmembrane domains
1580 which allow association with other members of the family and with other
1581 proteins to generate dynamic membrane domains.

1582

1583 **GlycosylPhosphatidyInostol (GPI)-anchored proteins**: a posttranslational
1584 modification, comprising a phosphoethanolamine linker, glycan core, and
1585 phospholipid tail, that anchors the modified protein to the outer leaflet of the
1586 cell membrane.

1587

1588 **lipid rafts:** specialized membrane microdomains enriched in cholesterol and
1589 glycosphingolipid which serve as organizing centers for the assembly of
1590 signalling molecules.

1591

1592 **KRAS–MEK signalling pathway:** interaction between the proto-oncogene
1593 KRAS which encodes a small GTPase and the downstream effector, the
1594 canonical RAF–MEK–EERK signalling pathway. Both pathways have roles in
1595 cell division, cell differentiation and apoptosis.

1596

1597 **Major Vault protein:** the main component of ribonucleoparticles termed
1598 vaults, which also contains two additional proteins, the vault poly (ADP-ribose)
1599 polymerase (vPARP) and the telomerase-associated protein 1 (TEP1), and
1600 several short untranslated, vault RNAs (vRNA). It has been implicated in the
1601 regulation of several cellular processes including transport mechanisms,
1602 signal transmissions and immune responses.

1603

1604 **Y-box protein 1:** a transcription factor shown to have a role in oncogenic cell
1605 transformation, multiple drug resistance and dissemination of tumours.

1606 **aminophospholipid translocases:** enzymes which transport
1607 phosphatidylserine and phosphatidylethanolamine from one side of a bilayer
1608 to another.

1609 **scramblases:** proteins responsible for the translocation of phospholipids
1610 between the inner and outer leaflet of a cell membrane.

1611 **calpain:** a calcium-dependent protein expressed ubiquitously in mammals
1612 and many other organisms.

1613

1614 **Rho family of small GTPases:** a family of small signalling G proteins
1615 implicated in the regulation of many aspects of actin dynamics.

1616

1617 **brush border:** the microvilli-covered surface of epithelial cells found in
1618 enterocytes in the intestine.

1619

1620 **Warburg effect:** an aerobic process whereby cancer cells produce energy by
1621 a high rate of glycolysis followed by lactic acid fermentation in the cytosol,
1622 rather than by oxidation of pyruvate in mitochondria

1623

1624 **zipcode RNA sequence motifs:** cis-acting regulatory sequences (25
1625 nucleotides) in the 3'-untranslated region (3'UTR) of mRNA transcripts that
1626 mediate binding of a ribonuclear protein complex to the mRNA, thereby
1627 temporarily blocking mRNA translation and that mediate movement of mRNA
1628 via the cytoskeleton to a cellular location where mRNA is released from
1629 protein binding and translation initiates.

1630

1631 **immunological synapse:** a specialized cell–cell junction between a thymus-
1632 derived lymphocyte (T cell) and an antigen-presenting cell.

1633

1634 **Hedgehog:** an essential signalling molecule, termed morphogen, required for
1635 numerous processes during animal development.

1636

1637 **ISGylation:** an ubiquitin-like modification that controls exosome release by
1638 decreasing the number of multivesicular endosomes.

1639

1640 **caveolin:** the principal component of caveolae, which are involved in receptor
1641 (clathrin)-independent endocytosis, mechanotransduction and lipid
1642 homeostasis.OK

1643

1644

1645 **SNARE proteins:** an acronym derived from "**SNAP** (Soluble NSF Attachment
1646 Protein) **RE**ceptor"); their primary role is to mediate the fusion of intracellular
1647 vesicles with their target membrane bound compartments.

1648

1649 **Synaptotagmin family:** family of membrane-trafficking proteins that has been
1650 implicated in calcium- dependent neurotransmitter release.

1651

1652 **Protein kinase C:** a serine/threonine kinase which plays important roles in
1653 several signal transduction cascades by controlling the function of other
1654 proteins through their phosphorylation.

1655

1656 **P2x₇ receptors:** trimeric ATP-gated cation channels found predominantly, but
1657 not exclusively, on immune cells, which have been implicated in various
1658 inflammatory, immune, neurologic and musculoskeletal disorders.

1659

1660 **follicular dendritic cells:** cells of the immune system found in primary and
1661 secondary lymph follicles of the B cell areas of the lymphoid tissue.

1662

1663 **lectin:** a carbohydrate-binding protein that is highly specific for sugar
1664 moieties.

1665

1666 **proteoglycans:** heavily glycosylated proteins consisting of a “core protein”
1667 with one or more covalently attached glycosaminoglycans (GAG) chains

1668

1669 **Intercellular adhesion molecules (ICAMs):** member of the immunoglobulin
1670 superfamily, which are involved in inflammation, immune responses and in
1671 intracellular signalling events.

1672

1673 **macropinocytosis** a form of regulated endocytosis that involves the non-
1674 specific uptake of extracellular material (such as small soluble molecules,
1675 nutrients, antigens) by invagination of the plasma membrane, which is then
1676 pinched resulting in small vesicles in the cytoplasm.

1677

1678 **trophoblast:** cells which form the outer layer of a blastocyst, provide nutrients
1679 to the embryo and give rise to a large part of the placenta.

1680

1681 **microglia:** a type of brain glial cell acting as the first and main endogenous
1682 immune defense in the central nervous system

1683

1684 **astrocytes:** star-shaped glial cells in the brain involved in nutrient supply,
1685 maintenance of extracellular ion balance and in tissue repair following brain
1686 injuries

1687

1688 **filopodia:** highly dynamic actin-rich cell-surface protrusions used by cells to
1689 sense their external environment.

1690

1691 **suicide mRNA/protein:** suicide gene which encodes nonmammalian
1692 enzymes that convert an inactive drug into highly toxic metabolites that inhibit
1693 the synthesis of nucleic acids and causes cells to go to apoptosis

1694

1695

1696

1697

1698 **Key points**

1699 • Secretion of extracellular vesicles was initially described as means of
1700 selective elimination of proteins, lipids and RNA from the cells. Now,
1701 extracellular vesicles are also considered as a new mode of
1702 intercellular communication.

1703 • In any given setting, population of extracellular vesicles comprises
1704 diverse subpopulations that can differ in size, morphology, composition
1705 or biogenesis mechanisms. Complementary methods of analysis are
1706 required to distinguish between these subpopulations.

1707 • Several machineries, prominently including components of the
1708 endocytic sorting machineries, act concomitantly for the generation of
1709 extracellular vesicles. In result, extracellular vesicles can largely vary in
1710 terms of their composition and may carry specific sets of proteins,
1711 lipids, RNA species that then determine their fate and functions.

1712 • Generation of extracellular vesicles requires a fine tuning of various
1713 intracellular trafficking processes, which define the composition of
1714 nascent vesicles and impact their generation and, in the case of
1715 exosomes, their secretion from an intracellular compartment.

- 1716 • Interactions of extracellular vesicles with recipient cells ~~and their~~ can
1717 have various effects on the target cell, from stimulating signalling
1718 pathways to providing trophic support, which depends on the mode of
1719 interaction and the intracellular fate of the vesicles in case of their
1720 uptake.
- 1721 • Studies of the cell biology of extracellular vesicles is not only essential
1722 for addressing cell biological questions but is also critical to open new
1723 avenues for their clinical use as biomarkers, cargo vehicles for targeted
1724 delivery of compounds or as specific modulators of cell behaviours.

1725

1726

1727 **Author biographies**

1728

1729 Guillaume van Niel, is a research director at CNRS and team leader of the
1730 Center of Psychiatry and Neurosciences, U894 INSERM. His research
1731 focuses on the processes and functions of endosomes and exosomes with
1732 implications in neuropathies using Zebrafish as an *in vivo* model system.

1733

1734 Gisela D'Angelo, is a research associated at CNRS, in Graça Raposo team,
1735 UMR144, at the Curie Institut. Her research interest is on the biogenesis and
1736 function of extracellular vesicles in developmental biology and cancer using
1737 *Drosophila epithelium* as an *in vivo* model system.

1738

1739 Graça Raposo, is a research director at CNRS, Deputy Director and team
1740 leader of the Department of Cell Biology, UMR144, in Institut Curie. Her
1741 research interest is on Intracellular trafficking, the biogenesis and functions of
1742 exosomes and lysosome related organelles such as melanosomes with
1743 implications lysosomal diseases and cancer.

1744

1745 **Author contributions**

1746 All authors contributed equally to all aspects of the article (researching data
1747 for article, substantial contribution to discussion of content, writing,
1748 review/editing of manuscript before submission).

1749

1750

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