

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

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## 14 **Abstract :**

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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**

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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<sup>1,2,3</sup>. Secretion of extracellular vesicles has been initially described as  
41 means of eliminating obsolete compounds<sup>4</sup> 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<sup>5,6,7</sup>.

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 means<sup>8-10</sup>. 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 degradation<sup>11</sup>) was initially used to name vesicles of  
59 an unknown origin released from a variety of cultured cells and carrying 5'-  
60 nucleotidase activity<sup>12</sup>. Subsequently, the term exosomes was adopted to  
61 refer to membrane vesicles (30-100 nm in diameter) released by **reticulocytes**  
62 **[G]** during differentiation<sup>4</sup>. 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<sup>13,14</sup> (Fig 1a-c). In the mid 1990's exosomes were reported to be  
67 secreted by B lymphocytes<sup>15</sup> and dendritic cells<sup>16</sup> 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 documented<sup>5</sup>.

72

73 Microvesicles, formerly called “platelet dust”, were described as subcellular  
74 material originating from platelets in normal plasma and serum<sup>17</sup>. Later,  
75 ectocytosis, a process allowing the release of plasma membrane vesicles,  
76 was described in stimulated neutrophils<sup>18</sup>. Although microvesicles were mainly  
77 studied for their role in blood coagulation<sup>19,20</sup>, more recently they were  
78 reported to have a role in cell–cell communication in different cell types,  
79 including cancer cells<sup>21</sup> 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 space<sup>22</sup> (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 compositions<sup>5</sup> (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 origin<sup>23</sup>.  
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 migration<sup>24</sup> or arrestin domain-  
97 containing protein 1-mediated microvesicles (ARMMS)<sup>25</sup>, 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).

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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<sup>26 27</sup>. 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.

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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 membrane<sup>10</sup>. 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 ILVs<sup>28</sup>. 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 membrane<sup>29</sup>.

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 them<sup>5</sup>.  
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 cargoes<sup>30</sup> (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 biogenesis<sup>31</sup>. 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 II<sup>32</sup> promotes MVE  
158 formation with a consequent release of extracellular vesicles, likely by  
159 recruiting sorting machineries that will promote MVE and ILV generation<sup>32,33</sup>.

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 maturation<sup>34</sup> (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 reticulocytes<sup>35</sup>. 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 recycling<sup>36</sup> and in the  
170 sorting of **syndecan [G]** in MVEs<sup>37</sup> 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  $\beta$ 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<sup>38,39</sup>. 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 cells<sup>40</sup>.

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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 ILVs<sup>41</sup>. 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 cells<sup>42</sup>.

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 targeting<sup>23</sup>  
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 exosomes<sup>43</sup>. 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)<sup>37</sup>.

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 complexes<sup>44</sup>.  
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 ceramide<sup>45</sup>. Ceramide may then allow  
229 generation of membrane subdomains<sup>46</sup>, which impose spontaneous negative  
230 curvature on the membranes. Alternatively, ceramide could be metabolized to  
231 sphingosine 1-phosphate to activate G<sub>i</sub>-protein coupled sphingosine 1-  
232 phosphate receptor that appears essential for cargo sorting into exosomal  
233 ILVs<sup>47</sup> (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<sup>48,49</sup>,  
237 in cargo (apolipoprotein E) targeting to exosomes secreted by melanoma  
238 cells<sup>50</sup> and in the biogenesis of exosomes in fibroblasts from down syndrome

239 patients<sup>51</sup>. Tetraspanins CD81, CD82 and CD9 are also directly involved in  
240 the sorting of various cargo to exosomes<sup>52,53</sup>. Mechanistically, these proteins  
241 form clusters and dynamic membrane platforms with other tetraspanins and  
242 with different transmembrane and cytosolic proteins<sup>54</sup> 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 enriched<sup>55</sup> (Fig 2). But tetraspanins also regulate the intracellular routing of  
249 cargoes towards MVEs, such as integrins<sup>56</sup>, 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<sup>57,58</sup>.  
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 membranes<sup>59</sup>. It has also  
266 been proposed that some cytosolic proteins, modified by ubiquitylation<sup>60</sup> or  
267 farnesylation<sup>61</sup> 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)<sup>62,63</sup>  
271 and DNA sequences<sup>64,65</sup>. Interestingly, miRNAs have been shown to be  
272 differentially sorted to exosomes, depending on their sequence (presence of



273 specific motifs)<sup>66</sup>, 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 unclear<sup>67</sup>. 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 complex<sup>68</sup>, the tetraspanin-enriched microdomains that could  
280 sequester many RNA-binding proteins in the membrane subdomains<sup>69</sup> or the  
281 miRNA-induced silencing complex (miRISC) and protein argonaute 2  
282 (AGO2), which mediate RNA silencing processes<sup>70</sup>. New regulators of miRNA  
283 sorting into exosomes have also recently been described and include the  
284 **KRAS–MEK signalling pathway [G]** acting through AGO2 <sup>71</sup>, **Major Vault**  
285 **protein [G]** <sup>72</sup> or **Y-box protein 1 [G]** <sup>73</sup>.

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 reticulocytes<sup>74</sup>, or  
292 during cell maturation as shown for dendritic cells<sup>75</sup>. Accordingly, most cells  
293 host subpopulations of MVEs distinguished by different lipid and protein  
294 compositions and morphology<sup>52,76</sup>. In this context, different sorting  
295 mechanisms can act on the same endosomal compartment<sup>49</sup> 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)<sup>52,77</sup>, 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)<sup>49</sup>. 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 MVE<sup>78</sup>;  
305 alternatively or concomitantly, distinct subpopulations of MVEs may exist and  
306 may be targeted by different machineries<sup>5,49</sup> (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<sup>5,6</sup>. 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.

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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 bodies<sup>79</sup>, 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 Ca<sup>2+</sup> levels<sup>31</sup>. Ca<sup>2+</sup>-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<sup>21,80</sup> (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 microvesicles<sup>80</sup>. However, even when the membrane lipid asymmetry is  
335 maintained, microvesicle biogenesis might proceed<sup>81,82</sup>. 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 neutrophils<sup>83</sup>.

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 cells<sup>84</sup>. 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 microvesicles<sup>85</sup>.

349

350 The biogenesis of tumor-derived microvesicles (oncosomes) is also tightly  
351 associated with metabolic changes, the so-called **Warburg effect [G]**<sup>86</sup>. In  
352 breast cancer cells, elevated glutaminase activity is dependent on Rho  
353 GTPases<sup>87</sup>, 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<sup>88,89</sup> — 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<sup>88,89</sup>. 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 microvesicles<sup>90</sup>, 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]**<sup>52,91</sup>. 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<sup>92,93</sup>. The impairment of lysosomal activity by  
410 inhibiting the endosomal proton pump V-ATPase also leads to an increase of  
411 exosome release<sup>94,95</sup>, and, for example, has been shown to trigger apical  
412 secretion of **Hedgehog [G]**-related peptides through a multivesicular  
413 compartment in *Caenorhabditis elegans*<sup>96</sup>.

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 secretion<sup>23</sup> and generally associated with degradative  
420 MVE, the syndecan–syntenin–ALIX pathway seems to be restricted so far to  
421 exosome secretion<sup>37</sup>. 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<sup>52,77</sup>. 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)<sup>94</sup>, or the tetraspanin 6<sup>97</sup>,  
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<sup>49,52,98</sup>.

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 secretion<sup>99</sup> (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 formation<sup>100</sup>. 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 proteins<sup>101</sup>. 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)<sup>102,103</sup>.

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<sup>52,104</sup>. 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)<sup>91</sup> (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 lysosomes<sup>105</sup>. Interestingly, Rab7 is also  
472 mandatory for the release of exosomes<sup>37</sup>. 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 secretion<sup>106</sup>. 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 exosomes<sup>107</sup>. 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 Rab27b<sup>32</sup> 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 cytoskeleton<sup>108</sup>, a step that is common to all mechanisms  
491 involving vesicular secretion. Rab27 also controls secretion of secretory  
492 lysosomes so called lysosome related organelles<sup>109</sup>, 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<sup>110,111</sup> 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]** members<sup>112</sup>.  
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 7<sup>113</sup>. This  
508 complex is involved in exosome secretion in some cells (human leukemia  
509 K562 cell line)<sup>114</sup> but not in others (MDCK cells)<sup>115</sup>. The process of exosome  
510 secretion has been demonstrated in several cell types to be regulated by Ca<sup>2+</sup>

511 <sup>116-118</sup>, 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 mastocytes<sup>119</sup> — also in exosome secretion<sup>120</sup>,  
514 strengthens the notion that MVEs are indeed specialized secretory organelles.  
515 Additional SNARE proteins involved in exosome secretion such as YKT6 <sup>121</sup> in  
516 *Drosophila*, SYX-5 in *C. elegans* <sup>122</sup> and syntaxin 1a <sup>123</sup> 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 cells<sup>95</sup> or the fraction of  
527 extracellular vesicles that can be recaptured by the same cell<sup>124</sup>. 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<sup>85,125</sup>. 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<sup>39 126 127</sup>. In HeLa cells another regulator of actin dynamics, Cdc42  
539 has been shown to be involved, but the underlying mechanism is still not  
540 known<sup>84</sup>. 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)<sup>25</sup>. 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 bilayer<sup>128</sup>. 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 release<sup>129</sup>. What is known is that a strong microvesicle  
555 release is induced by increased concentration of  $\text{Ca}^{2+}$ , 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 esters<sup>130</sup>. Release of microvesicles has also  
559 been shown to depend on ATP-mediated activation of **P2x<sub>7</sub> receptors [G]**,  
560 which leads to rearrangements of the cell membrane<sup>131,132</sup>. 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 shedding<sup>133</sup>. 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 exosomes<sup>45</sup> (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 vesicles<sup>134</sup>. 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]**<sup>135</sup>, intestinal epithelial cell<sup>136</sup>, dendritic cells<sup>137</sup>, or  
593 neurons<sup>138</sup>, and also in liver, lungs or lymph nodes<sup>136,139</sup>. Of note, the  
594 recipient cells can also be the producing cell itself, leading to autocrine  
595 responses<sup>124</sup>.

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)<sup>140</sup> 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<sup>141,142</sup> and  
606 microvesicle<sup>143</sup> 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 organs<sup>139</sup>. 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 composition<sup>139</sup>. Exosomal tetraspanins could also regulate cell targeting. They  
613 have been shown to interact with integrins<sup>144</sup> and to promote exosome  
614 docking and uptake by selected recipient cells<sup>145,146</sup>. Other molecules such as  
615 heparan sulphate proteoglycan and lectins, both present in extracellular  
616 vesicles and at the plasma membrane, contribute to their docking and/or  
617 attachment of these vesicles to recipient cells (Fig 5). Glypican-1, a cell  
618 surface proteoglycan that bears heparan sulfate, and CD44, a cell-surface  
619 glycoprotein involved in cell–cell interactions, are involved in exosome<sup>147</sup> and  
620 microvesicle<sup>148</sup> 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<sup>140 149,150</sup> 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<sup>135, 52</sup> or may be internalized by clathrin-mediated or  
629 clathrin-independent endocytosis, such as **macropinocytosis [G]** and  
630 phagocytosis<sup>151-153</sup> as well as through endocytosis via caveolae and lipid  
631 rafts<sup>157-159</sup> (Fig 5 ). Of note, certain cell types, such as HeLa cells or EBV-  
632 transformed B cell line release clusters of exosomes, as a result of tethering  
633 the vesicles by protein tetherin<sup>95</sup>. This clustering may affect the way these  
634 vesicles are internalized, favouring phagocytosis or macropinocytosis to  
635 support the engulfment of such large masses or aggregates of extracellular  
636 vesicles<sup>151</sup>.

637

638 Specific composition of extracellular vesicles will influence their fate. The  
639 presence of Amyloid precursor protein on one exosome subtype from  
640 neuroblastoma cells will specifically target them to neurons contrary to a  
641 CD63 enriched exosome subtype that binds both neurons and glial cells<sup>154</sup>.  
642 Another example is the presence of syncytin at the surface of exosomes  
643 derived from the **trophoblast [G]** that promotes their uptake<sup>155</sup>, 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 monocytes<sup>156</sup>.

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]**<sup>157</sup>. It has also been shown that **filopodia [G]** drive  
653 extracellular vesicles toward sites of uptake<sup>158</sup>. 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 vesicles<sup>159</sup>.

657

658 Following interaction with the plasma membrane of recipients cells<sup>157</sup> 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<sup>160,161</sup>. 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 cell<sup>162</sup>, 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)<sup>155</sup>.

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<sup>15,16</sup>. Tumour derived microvesicles were shown to carry  
681 fibronectin, which when bound to integrin on non-transformed fibroblasts was  
682 able to promote their anchorage independent growth (one of the hallmarks of  
683 tumorigenesis), contributing to the acquisition of transformed phenotype by  
684 healthy cells<sup>163</sup>. As another example, microvesicles generated and released  
685 by embryonic stem cells were shown to induce invasion of maternal tissue by  
686 the trophoblast, which is mediated by the interaction of laminin and fibronectin  
687 on the microvesicles with integrins along the surfaces of the trophoblast, and  
688 which promotes embryo implantation<sup>164</sup>. The role of extracellular vesicles in  
689 the long-range transfer of morphogens to recipient cells in developing  
690 organisms was also shown<sup>165</sup>.

691

692 Cargo delivered by extracellular vesicles can also activate various responses  
693 and processes in the recipient cell after internalization. For example, in  
694 dendritic cells, protein cargo of exosomes derived from intestinal epithelial  
695 cells<sup>136</sup> or other dendritic cells<sup>140</sup> is processed in the endocytic compartment  
696 similarly to antigens and then used in antigen presentation, thereby  
697 contributing to immune response regulation. Extracellular vesicles could also  
698 fuse directly with the plasma membrane or with the endocytic membrane of  
699 recipients cells. Such processes are mandatory to release intraluminal content  
700 in the cytoplasm of recipient cells, a key step to support the release of  
701 miRNA<sup>62</sup> and mRNA<sup>166</sup> from extracellular vesicles into recipient cells to  
702 regulate gene expression. Direct fusion of extracellular vesicles with the  
703 membrane of recipients cells allow also the exchange of transmembrane  
704 proteins and lipids. Extracellular vesicles can transport various lipid species  
705 including eicosanoids, fatty acids, and cholesterol as well as lipid  
706 translocases, thereby contributing to the regulation of bioactive lipid  
707 species<sup>167</sup>. 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 amyloids<sup>168</sup>.

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 viruses<sup>169</sup>.

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 cells<sup>170</sup> (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 others<sup>6</sup>. 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<sup>139 171</sup>, or  
731 particular components associated with neurodegenerative diseases<sup>172</sup>. 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 documented<sup>163</sup>. 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 tissues<sup>173</sup>.

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 diseases<sup>172</sup>. 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 biomarkers<sup>174</sup>. 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 responses<sup>175</sup>. 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<sup>176,177</sup>. 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 cancers<sup>178</sup>. 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<sup>5,179,172</sup>). 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 infection<sup>180</sup>. Such strategy could be  
778 considered for fungi, bacteria, parasitic protozoa and helminths<sup>172</sup>.  
779 Mesenchymal stem cells-derived extracellular vesicles are now tested in  
780 animal models to treat acute kidney failure<sup>181</sup>, myocardial infarction<sup>182</sup> or

781 ischemia<sup>183</sup>. 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<sup>184,185</sup>. 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 brain<sup>184</sup>.

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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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<sup>67,186</sup>. 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 studies<sup>187</sup>. 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 limitations<sup>188</sup> but allows the tracking of extracellular vesicle by live cell  
1389 imaging in cell lines and, to limited extent, also *in vivo*<sup>189</sup>. 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)<sup>190,191</sup> 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 cells<sup>157</sup>. 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)<sup>95,124</sup>. 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  $\mu\text{m}$ ). 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<sup>23,192</sup>. 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<sup>193 194 195</sup>. 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<sup>52 74</sup>, on  
1499 the post-translational modification of the cargo (for example: proteolytic  
1500 processing, ubiquitylation)<sup>49,193</sup> 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 **P2x<sub>7</sub> 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  
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1733

1734 Gisela D'Angelo, is a research associated at CNRS, in Graça Raposo team,  
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1738

1739 Graça Raposo, is a research director at CNRS, Deputy Director and team  
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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

1751 **Competing interests statement**

1752 The authors declare no competing interests.

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