Chapter

# Mechanisms of Extracellular Vesicle Biogenesis, Cargo Loading, and Release

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## Abstract

Extracellular vesicles (EVs) are carriers of various biomolecules including bioactive enzymes, lipids, proteins, nucleic acids, and metabolites. EVs are classified into three main types based on their size, biogenesis, and cargo. Exosomes originate from endosomal membranes and are the smallest type of EV. Microvesicles (MVs) or microparticles are larger in size, and like apoptotic bodies which represent the largest type of EVs, both of these vesicles originate from outward budding of the plasma membrane. As discussed in this chapter, cargo loading of EVs and their release into the extracellular space where they can be taken up by neighboring or distant cells plays an important role in physiology and pathophysiology. This chapter will outline specific mechanisms involved in the loading and enrichment of miRNAs, proteins, and lipids within EVs. As explained here, various external and biological stimuli play a role in EV release. Finally, recent studies have shown that the biogenesis, cargo loading, and release of EVs are governed by circadian rhythms. Although EVs were once thought to serve as garbage disposals of cells, the numerous roles they serve in physiology and pathophysiology are now being appreciated.

**Keywords:** extracellular vesicles, exosomes, microvesicles, microparticles

## 1. Introduction

Extracellular vesicles (EVs) represent a heterogenous population of vesicles that include exosomes and the plasma membrane shedding microvesicles (MVs) (also known as microparticles) and apoptotic bodies. Exosomes represent the smallest subtype of EVs and have spherical bodies with a lipid bilayer membrane. Exosome formation begins in endosomes as the budding of the endosomal membrane results in the formation of multivesicular bodies (MVBs) [1]. The fusion of MVBs with the inner leaflet of the plasma membrane results in the release of intraluminal vesicles (ILVs) as exosomes (**Figure 1**) [1]. The ILVs within the lumen of the endosomes have three fates [2]. First, the contents can be used for the biogenesis of specialized lysosome-related organelles such as melanosomes. Second, the ILVs may fuse with lysosomes. Third, the ILVs may fuse with the plasma membrane to release the content

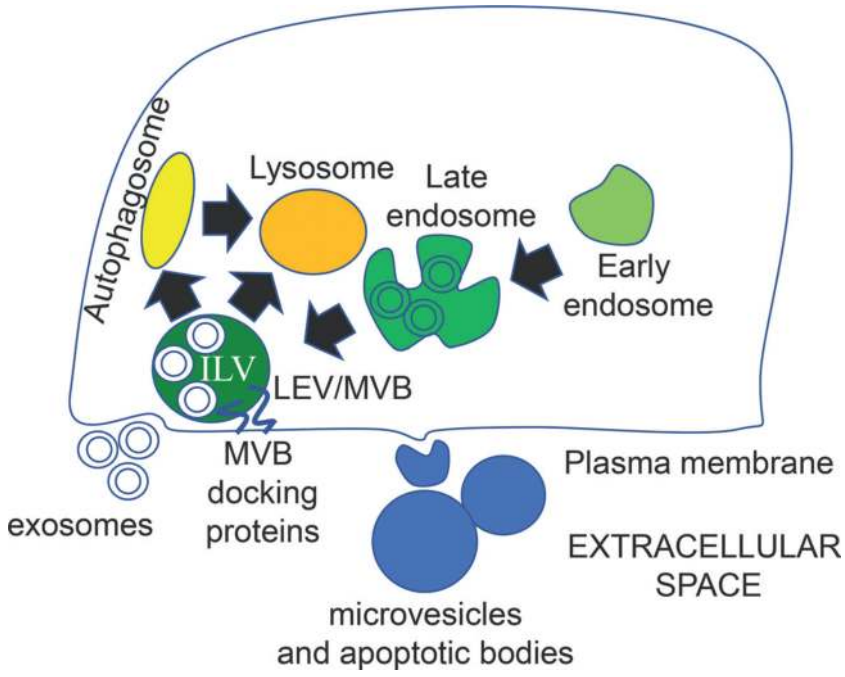


**Figure 1.**
*Biogenesis of EV's. Microvesicles and apoptotic bodies are generated by outward budding of the cell's plasma membrane. Exosomes are formed from the endocytic pathway of the cell. In this process early endosomes undergo inward budding to form intraluminal vesicles (ILVs) inside the late endosomal vesicles (LEVs) or multivesicular bodies (MVBs). The fate of the MVBs include degradation by lysosomes, fusion with autophagosomes, or fusion with the plasma membrane of the cell resulting in the release of the ILVs to the extracellular space in the form of exosomes. As shown, MVBs and autophagosomes can be degraded by lysosomes.*

into the extracellular space in which the vesicles will then be termed exosomes. There are a number of signaling and specialized proteins that contribute to the biogenesis of exosomes. The biogenesis of microvesicles and apoptotic bodies is different from that of exosomes as these vesicles are produced from the shedding or budding of the plasma membrane from the parent cell (**Figure 1**). The release of EVs from a cell can be triggered by a myriad of stimuli. Importantly, not all EVs are created equally and EVs found in biological fluids including plasma and urine represent a heterogenous mixture of subpopulations of EVs. The fate of EVs after they are released from their parent cell is to either be taken up by a neighboring or distant cell or to be excreted from the body.

## 2. ESCRT dependent and independent multivesicular body biogenesis

Endosomal sorting complex required for transport (ESCRT) proteins and ESCRT associated proteins including TSG101, ALG-2 interacting protein X (ALIX), SKD1, and Chmp4 are required for ESCRT dependent MVB biogenesis. ESCRT is a ubiquitin-dependent mechanism that contributes to the sorting of ubiquitinated proteins into exosomes. ESCRT consist of four complexes that are numbered according to their sequential action. For example, ESCRT-0 aka vacuolar protein sorting (VPS) associated protein 27/heat shock element 1 complex VPS27/HSE1 recruits ESCRT-1, which plays an essential role in MVB cargo sorting and bud formation. VPS36 associates with ESCRT-1 via a ubiquitin moiety while VPS25 is required for the assembly of the ESCRT-II complex [3]. ESCRT-II also plays a role in cargo sorting and regulates

ESCRT-III formation. VPS20 is involved in the assembly of the ESCRT-III complex and VPS24 completes the scission of the budding membrane [4].

In addition to sorting of cargo within multivesicular bodies, ESCRT-III participates in ESCRT recycling and binds ALIX to allow for cargo sorting [5]. ALIX also stimulates exosome secretion containing the tetraspanins CD63, CD9 and CD81 [5].

Alternatively, there are multiple ESCRT independent MVB biogenesis pathways that utilize various other proteins. This particular mechanism may be dependent on alternate mechanisms including the Rab associated proteins, ceramides, sphingomyelins, cholesterols, and tetraspanins. Members of the Rab family of small GTPases regulate various steps in the formation and transition of the endosomal transport network. Rab5 is thought to regulate the formation and fusion of early endosomes [6, 7]. Rab7 is thought to regulate the fusion of late endosomes/multivesicular endosomes with lysosomes resulting in degradation of the intraluminal vesicles [8, 9]. Rab27 is thought to regulate multivesicular endosome docking and fusion with the plasma membrane to allow for release of the intraluminal vesicles as exosomes [10]. One report provided evidence that Rab31 engages flotillin proteins from lipid rafts to orchestrate epidermal growth factor receptor entry into multivesicular endosomes to form intraluminal vesicles (ILV) and the release of exosomes [11].

## 3. Lipid rafts and exosome biogenesis

There are a number of lipid raft proteins that are associated with exosome biogenesis. Ceramide is enriched in the inner leaflet of the exosomal membrane [12]. One feature of ceramides is that they can initiate spontaneous membrane invagination which allows for ILV formation and the maintenance of vesicle shape and structure. One study showed exosome release was reduced after the inhibition of the enzyme that catalyzes the formation of ceramide from sphingomyelin, neutral sphingomyelinases [13]. Cholesterol is another lipid raft protein that is enriched in the exosomal membranes. The accumulation of cholesterol leads to the secretion of exosomes enriched in flotillins. In some cells the inhibition of sphingomyelinase results in the suppression of exosome production while exosome production is not affected by changes in sphingomyelinase activity in other cells. This suggest that there may be cell type specific mechanisms that regulate exosome production. For example, the ESCRT dependent mechanism may be favored over the lipid raft dependent mechanism in PC-3 cells since exosome production is not affected by inhibition of sphingomyelinase in these cells.

There are several other proteins that are involved in exosome biogenesis. Proteins associated with the ESCRT dependent pathway, glycosylphosphatidylinositol anchored proteins (GPIAPs), palmitoylated forms of transmembrane proteins, flotillins, annexins, and cholesterol binding proteins such as caveolins have been shown to be enriched in exosomes. Flotillin 1 and Flotillin 2 appear to play a role in EV composition as Phuyal et al. showed interfering RNA-mediated knockdown of flotillin 1 and flotillin 2 resulted in alteration of the EV composition in PC-3 cells [14]. The exosomal protein Annexin A2 may play an essential role in the organization of the exosomal membrane as it preferentially binds to phosphatidylinositiol 4,5 bisphosphate rich domains and cholesterols within the cytoplasmic leaflet of lipid raft membranes and influence raft dynamics of parent cells [15]. It is not surprising caveolins were found to play an essential role in EV generation and uptake since they are known to regulate multiple cellular processes including endocytosis, exocytosis,

and maintaining the shape of the cell membrane. Accumulating data suggest caveolin 1 promotes the production and release of EVs while caveolin deletion results in a decrease in EV release [16].

## 4. Regulation of size distribution

The size of EVs may or may not depend on the amount and type of cargo enriched in the vesicles. Some studies have shown an increase in EV release without a change in EV size, while other studies have shown the contrary [17, 18]. Apoptotic bodies represent the largest type of EVs and contain organelles, in addition to nucleic acids, proteins, and lipids. Conversely, exosomes and microvesicles lack organelles, but contain specific molecules of interest.

Numerous studies have shown the same cell type can produce subpopulations of exosomes that are remarkable different in size. For example, polarized epithelial cells, such as those in the kidney tubule release exosomes across the apical plasma membrane and the basolateral plasma membrane (**Figure 2**). These two types of exosomes show distinct differences in their size and protein composition [19]. Interestingly, Matsui et al. showed two independent mechanisms for exosome release across the apical and basolateral plasma membranes [20]. This group showed that ceramide plays an essential role in basolateral exosome release whereas, ALIX is important for apical exosome release [20].

Although it is reasonable to expect the size of EVs to be directly proportional to the concentration of a particular molecule present in the EVs, this is not always the case. For example, Chacko et al. showed urinary EV concentration decreased after infusing Tempol in hypertensive 129Sv mice while the size of the EVs increased [21].

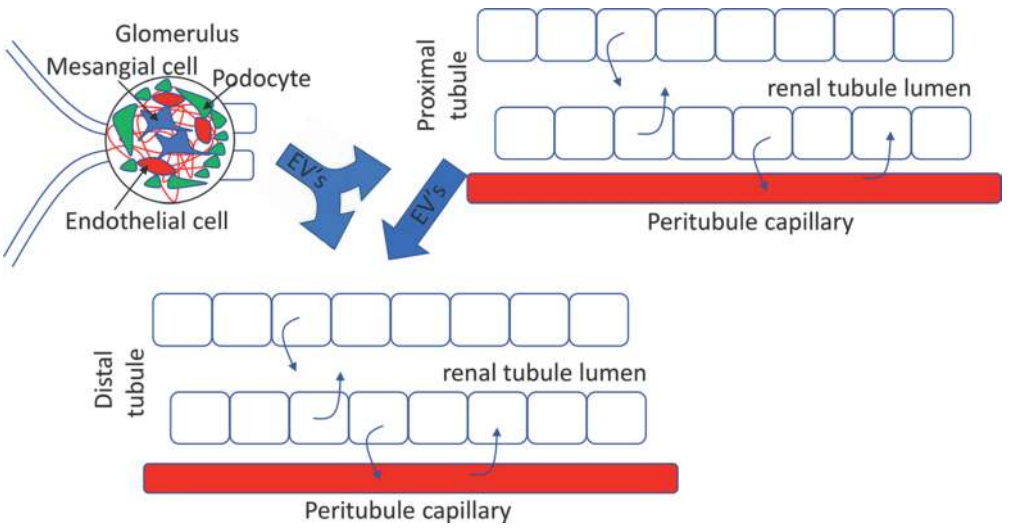


**Figure 2.**
*Release of EV's from various kidney cell types. All cell types in the kidney including glomerular cells and cells within each segment of the nephron release EV's that can allow for intercellular communication, intracellular signaling, and the regulation of various mechanisms in health and disease. Polarized epithelial cells of the nephron release two distinct populations of EV's across the apical membrane that faces the lumen and the basolateral membrane that faces the peritubular capillaries. EV's that are released into the lumen containing the filtrate can be taken up by recipient cells downstream in the nephron or excreted from the body in the form of urine. EV's that are released across the basolateral membrane can be reabsorbed back into the blood.*

The amount of the EV marker protein annexin A2, but not the amount of other EV marker proteins including TSG101 or flotillin-2 decreased after tempol treatment [21].

## 5. Cargo loading/sorting

The cargo that is loaded and carried within EVs include proteins, lipids, nucleic acids, metabolites, and miRNAs. The loading of specific molecules within different types of EVs is dependent on the parent cell and specific loading mechanisms. The enrichment of various types of cargo molecules is also dependent on a particular cell type being subject to pathological conditions, drugs, and various stimuli.

The loading of cargo within EVs may be a means of the cell getting rid of unneeded or excess material. For example, EVs from epithelial cells of the renal tubule are released across the luminal plasma membrane into the filtrate and are subsequently excreted from the body in the form of urine if the EVs are not taken up by recipient cells downstream in the nephron. In fact, a large amount of urinary EVs (uEVs) are those released from renal epithelial cells and bladder cells.

Multiple mechanisms have been proposed for the loading of miRNAs into EVs. One mechanism involves 3′ end uridylation of miRNAs [22]. A second mechanism involves the incorporation of a four nucleotide motif (GGAG) and its interaction with the ribonucleoprotein hnRNPA2B1 [22]. A third mechanism involves the RISC complex associated protein, AGO2 [22]. Another mechanism involves over-expression of nSMase2 [22]. The exosomal protein Annexin A2 was found to play a role in the packaging of miRNAs in EVs [23].

Proteins found to be enriched in EVs include those enriched in lipid rafts, metalloproteinases, tetraspanins, proteins associated with endosomes, heat shock proteins, glycosylphosphatidylinositol (GPI) anchored proteins, and those associated with the exosome biogenesis pathway. Proteomic studies have identified metalloproteinases including ADAMs (a disintegrin and metalloproteinase) with proteolytic activity enriched in EVs [24]. ADAMs are versatile proteins that are involved in cell adhesion, cellular communication, migration, and the regulation of growth factors and cytokines. EVs also contain members of the LAMP family proteins, LAMP-1 [25] and LAMP-2 [26]. These lysosome-associated proteins help maintain the integrity of the lysosomes by forming a glycocalix that protects the membranes from enzymatic degradation. Tetraspanins including CD9, CD63, and CD81 are enriched in exosomes [24]. Heat shock proteins (HSP) including HSP60, HSP70, and HSP90 have been found to be enriched in EVs [24]. Flotillin proteins were found to regulate exosome formation and cargo sorting. Other proteins such as syntenin has been found to contribute to the loading of exosomes with specific cargo [27]. GPI proteins including the complement regulator proteins CD55 and CD50 are enriched in exosomes [28].

Lipids that are enriched in EVs generally share the same features as the cells of origin. Lipids enriched in EVs include cholesterol, ceramides, cholesterol, and phosphatidylserine, which is also enriched in vesicles derived from MVBs. In one study, Glover found 13 ceramides that were significantly reduced in uEVs of hereditary α-tryptasemia (HαT) patients compared to healthy volunteers [29]. In another study, Nouri et al. showed sphingomyelin, phosphatidylethanolamine, and lysophosphatidylcholine were present in greater concentrations in EVs isolated from the conditioned media of human aortic endothelial cells compared to control EVs isolated from the complete growth media of these cells [18].

## 6. Incorporation of viral components into EVs

Accumulating experimental evidence suggest viral components are packaged in EVs during infection and this results in alterations within recipient cells. Virus proteins including nucleoproteins and glycoproteins packaged in EVs were found to induce apoptosis in recipient immune cells.

One study reported the presence of ebola virus VP40, nucleoprotein, and glycoprotein in EVs that leads to apoptosis in recipient cells [30]. Studies by the same group also showed that VP40 can become incorporated into exosomes and thereby negatively impact recipient T cells and monocytes [30–32]. Multiple groups showed Zika virus infected cells release EVs with infectious viral RNA and viral proteins [33, 34]. York et al. showed that Zika virus modifies EV density, cargo, and secretion [33]. Ning et al. showed RNA from SARS-CoV-2, the causative agent of Severe Acute Respiratory Syndrome, COVID-19 in plasma EVs just one day after infection which plateaued from day 6–28 in non-human primates and 20–60 days in young children [35]. In another study, Troyer et al. showed EVs released from cells expressing the CoV-2 spike protein contain multiple peptides originating from this protein [36]. This group reported EVs carrying these spike proteins can serve as a decoy for anti-spike neutralizing antibodies and therefore promote viral infection.

The pathogenic and deleterious effect of EVs containing viral components is not limited to acute disease but it can result in asymptomatic or recurrent infections. The delivery of various molecules packaged in EVs and the delivery of these EVs to healthy cells may allow the virus to remain latent.

## 7. EVs in promoting infectious disease

Protozoan parasites such as Leishmania, Plasmodium, and Toxoplasma contribute to significant morbidity and mortality in humans. Studies have shown EVs can modulate host immune cells [37]. *Leishmania* parasites are transmitted by sandflies and are responsible for infecting phagocytic cells inside the mammalian host. A study by Atayde et al. showed exosomes secreted within the midgut of *L. major* parasites enhance infection and contribute to the development of lesions after being injected in mice [38]. *Plasmodium* parasites are the etiological agents of malaria. Multiple studies have demonstrated an important role for EVs in *Plasmodium* infections. One study showed malaria-infected red blood cells use exosome-like vesicles to communicate which promotes differentiation to sexual forms [39]. Another study showed malaria antigens are enriched in microvesicles released from infected red blood cells which activate host monocytes and neutrophils [40]. *Toxoplasma* is acquired by ingestion of raw or uncooked meat and these parasites are common in virtually all species of warm-blooded vertebrates. Li et al. performed an analysis of differential exosomal miRNAs in dendritic cells induced by Toxoplasma gondii infection [41]. These studies also implicate a role for exosomes in toxoplasmosis and the ability to stimulate naïve recipient cells.

## 8. EV release in health and disease

A myriad of different mechanisms have been shown to contribute to EV release in various types of cells. These mechanisms include specialized roles of proteins

including Rab proteins, SNARE proteins, small GTPases of the Rho/Rac/cdc42 family, and diacyl glycerol kinase α. These mechanisms also include posttranslational modifications of EV cargo proteins, inhibition of various kinases, and activation of cell surface receptors. Different Rab proteins play specialized roles in regulating exosome transport between different cellular compartments. For example, Rab5 and Rab7 are important in delivering cargo to the early endosomes, while Rab27 is involved in membrane docking to promote fusion, while Rab11 and Rab35 are involved in MVE secretion [42]. The process by which exosomes bud off the plasma membrane is accomplished with the activation of the ARF6 protein [43].

The specific inhibition of protein kinase C, but not protein kinase D in cultured human aortic endothelial cells resulted in both an increase in EV size and increase in EV release [18]. It is still not known whether the increase in EV release is a direct consequence of decreased Protein Kinase C (PKC) activity or a indirect consequence resulting from other proteins that are regulated by PKC activity. Also, it is likely that various mechanisms that regulate PKC activity such as diacylglycerol (DAG) also regulate EV size and release. This could provide a feedback mechanism by which EVs that are released by one cell type and taken up by another cell type can regulate the release of EVs in the recipient cells.

Pharmacological activation of specific G-protein coupled receptors (GPCRs) from trophoblast cells was shown to trigger the release of EVs [17]. Activation of CCKBR, TAS2R14, cholinergic muscarinic 1 and 3, and angiotensin II receptors, each increased EV release without affecting the overall size of the EVs [17]. Also, EV release by the calcium ionophore, A23187, was less robust when compared to receptor-mediated stimulation [17]. These finding warrant the investigation on whether activation of other GPCRs can mediate EV release.

## 9. EV release by various stimuli

EV release can be triggered by several types of stimuli. Many of these stimuli alter exosome release in an ADAM dependent manner. For example, EV release by p53 activation in tumor cells during radiation treatment has been reported [44]. Bacterial toxins including lipopolysaccharide have been shown to enhance the release of exosomes enriched in ADAM17 [24]. Calcium mobilization by treatment with calcium ionophores such as ionomycin has been reported to activate ADAM10 and trigger the secretion of EVs [24]. On the other hand, PMA has been reported to activate ADAM17 and induce its enrichment in exosomes [24]. Hypoxia has been reported to induce metalloproteinases and the release of EVs [24].

Akuthota et al. showed human eosinophils which are known to secrete chemokines, cytokines, and cationic proteins also secrete MVs, and the secretion of these vesicles increase in response to inflammatory stimuli such as tumor necrosis factor alpha (TNF-α) stimulation and eotaxin-1 (CCL11) [45]. In another study, Hunter et al. showed MVs are released from human brain microvascular endothelial cells in response to either thrombotic or inflammatory stimuli in a sex dependent manner [46]. Experimental evidence suggests nitric oxide (NO) negatively regulates EV release [47].

Numerous studies have demonstrated oxidative stress regulates the release of EVs from various cell types. One study showed oxidative stress triggers the release of microparticles by human alveolar cells and human bronchial epithelial cells [48]. Another study showed oxidative stress induces the release of membrane complement

regulatory protein positive microparticles and this was blocked by the thiol antioxidant N-acetylcysteine amide [49]. Another study showed cigarette smoke extract induces exosome release by airway epithelial cells by depleting cell surface thiols but this is prevented by N-acetyl-L-cysteine and glutathione [50]. In another study, ATP mediated signaling through the purinergic P2X7 receptor was found to trigger macrophage activation of tissue factor activation procoagulant EVs and this was blocked by the ROS inhibitor N-acetyl cysteine or the flavoenzyme inhibitor diphenyleneiodonium but not nitric oxide synthase inhibitors [51]. In another study, elevated levels of carbon dioxide were reported to activate mitochondrial ROS in neutrophils resulting in an increase in microparticles release [52].

## 10. Circadian regulation of EVs

Circadian rhythms are seen in many physiological processes in several organ systems of various species and these rhythms are controlled by a master pacemaker located in the hypothalamus called the central suprachiasmatic nucleus and peripheral clocks in peripheral tissues. The circadian clocks within peripheral tissues not only regulate local physiological functions but they also contain essential core clock proteins such as Per1/2, Bmal1, Cry1/2, and Clock that work in concert to generate cell-autonomous oscillations and circadian rhythms.

Since the content of EVs represents a "snapshot" of a cell's internal environment at a given time, it is important to consider the regulation of EV biogenesis and cargo in a circadian dependent manner. Proteomic analysis of uEVs revealed these vesicles are enriched in at least 19 proteins that are associated with various renal diseases [53]. Proteins that were previously found to be packaged within uEVs include aquaporin-2, subunits of ENaC, the sodium chloride co-transporter (NCC) and the sodium potassium chloride co-transporter (NKCC2) [53–55]. Multiple studies have shown several of these proteins including ENaC and NCC are regulated by circadian clock proteins that are responsible for regulating circadian patterns and rhythm [56, 57].

At least in healthy male rats, the excretion of EVs into the urine appears to follow a circadian pattern [58]. This study reported that the highest excretion rate of EVs occurred during the active dark cycle (19,00–23,00). Additionally, the excretion rate of the EV associated protein TSG101 and the EV excretion rate showed a similar stoichiometry and circadian pattern. This finding suggest TSG101 can be used as a means of normalization of uEVs during time of day urine collections. The regulation of EV biogenesis and release in a circadian dependent manner for pathophysiological conditions is still currently under investigation. These studies are more complex since there are usually several variables associated with disease mechanisms that contribute to EV cargo loading and release.

## 11. Conclusions

Although EVs were once thought to serve as a garbage disposal system for cells, EVs are now regarded as important vehicles to shuttle biomolecules and allow for intercellular communication, cellular differentiation, intracellular signaling, and various other biological functions. The development of state-of the art equipment including advanced mass spectrometers for conducting lipidomic, proteomic, and

metabolomic studies coupled to bioinformatics for performing pathway analysis has led to a better understanding for the role of EVs in physiology and pathophysiology. The identification of packaged molecules within EVs is not only important for biomarker discovery and the identification of novel drug targets, but it also provide clues to answering other questions related to molecular mechanism. For example, as discussed earlier, various protozoan parasites are able to hijack the host cellular machinery to promote their own survival and propagation. The use of engineered EVs to deliver small molecule drugs is gaining attention as it represents an efficient mechanism to treat various diseases including cancers and metabolic diseases.

Although several fundamental questions surrounding the biogenesis, loading, and release of EVs have been answered within the past 10 years there are several questions that remain. First, the specific mechanisms that regulate the balance between the release of exosomes and the fates of ILVs remain largely unknown. Second, EVs may have a dichotomous role in either inhibiting or promoting viral infection, but there are unanswered questions for each distinct process. Third, mechanisms for the cell type specific recognition and uptake of EVs is not completely understood.

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## Conflict of interest

The author declare no conflict of interest.

## Appendices and nomenclature

Non-standard abbreviations:

| | |
|---|---|
| ADAM | a disintegrin and metalloproteinase |
| DAG | diacylglycerol |
| ESCRT | endosomal sorting complex required for transport |
| EVs | Extracellular vesicles |
| GPCRs | G-protein coupled receptors |
| GPIAP | glycosylphosphatidylinositol anchored proteins |
| HSPs | Heat shock proteins |
| ILVs | intraluminal vesicles |
| MVs | microvesicles |
| MVBs | multivesicular bodies |
| NO | nitric oxide |
| NCC | sodium chloride co-transporter |
| NKCC2 | sodium potassium chloride co-transporter |
| PKC | protein kinase C |
| TNF-α | tumor necrosis factor alpha |
| VPS | vacuolar protein sorting |

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