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Impact of Lysosome Status on Extracellular Vesicle Content and Release

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Abstract

Extracellular vesicles (EVs) are nanoscale size bubble-like membranous structures released from cells. EVs contain RNA, lipids and proteins and are thought to serve various roles including intercellular communication and removal of misfolded proteins. The secretion of misfolded and aggregated proteins in EVs may be a cargo disposal alternative to the autophagy-lysosomal and ubiquitin-proteasome pathways. In this review we will discuss the importance of lysosome functionality for the regulation of EV secretion and content. Exosomes are a subtype of EVs that are released by the fusion of multivesicular bodies (MVB) with the plasma membrane. MVBs can also fuse with lysosomes, and the trafficking pathway of MVBs can therefore determine whether or not exosomes are released from cells. Here we summarize data from studies of the effects of lysosome inhibition on the secretion of EVs and on the possibility that cells compensate for lysosome malfunction by disposal of potentially toxic cargos in EVs. A better understanding of the molecular mechanisms that regulate trafficking of MVBs to lysosomes and the plasma membrane may advance an understanding of diseases in which pathogenic proteins, lipids or infectious agents accumulate within or outside of cells.

Keywords

Alzheimer’s disease; HIV AIDS; Niemann Pick disease; Parkinson’s disease

Introduction

Extracellular vesicles (EVs) are small (30 – 150 nm diameter) vesicles secreted by most, if not all, cell types and present in blood, urine and cerebrospinal fluid (Colombo, Raposo et al. 2014). The secretion of EVs is highly conserved and has been demonstrated in bacteria (Kulkarni and Jagannadham 2014, Kaparakis-Liaskos and Ferrero 2015), protozoa (Mantel and Marti 2014), insects (Beckett, Monier et al. 2013), plants (An, van Bel et al. 2007, Mu, Zhuang et al. 2014, Samuel, Bleackley et al. 2015) and mammals (Colombo, Raposo et al. 2014). There are several categories of EVs: apoptosomes that are released from cells undergoing apoptosis, microvesicles that are released by evagination of the plasma membrane, and exosomes which are generated by the fusion of multivesicular bodies (MVB) with the plasma membrane and the release of intraluminal vesicles (ILV) into the extracellular fluid (Figure 1) (Colombo, Raposo et al. 2014, Yanez-Mo, Siljander et al.

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2015). The origin of exosomes suggests that they are most likely related to the endosomal-lysosomal pathway. Current methodologies are unable to distinguish between exosomes and microvesicles following their release as they are probably released from the same cells at the same time. Thus, most reports that use the terms exosomes or microvesicles actually measure the entire EV pool (Lotvall, Hill et al. 2014). Moreover, there are likely other subgroups of EVs based on the mechanism of secretion and on their content. In this review we will use the term EVs for isolated vesicles whose origin within the cell is unknown, and exosomes or microvesicles for studies of vesicles biogenesis where the EV origin is known. In all organisms, EVs contain a unique signature of lipids, membrane proteins, soluble proteins, RNA and, in some cases, DNA (Yanez-Mo, Siljander et al. 2015). It has been shown that these proteins, lipids, and RNA are actively sorted into EVs and the level of any given metabolite in EVs can be substantially different from its level in the parent cells. This complex cargo repertoire indicates that EVs have specific physiological roles that are just beginning to be understood (Yanez-Mo, Siljander et al. 2015).

Recent research has shown that EVs participate in processes ranging from immune function (Robbins and Morelli 2014), to stem cell biology (Lai, Yeo et al. 2015), metabolic regulation (Milbank, Martinez et al. 2016) and communication between neurons and glial cells in the nervous system (Fruhbeis, Frohlich et al. 2012, Yanez-Mo, Siljander et al. 2015). Moreover, beyond serving as intercellular signaling vessels, EVs can also affect their parent cells by removing proteins from their surface or interior (Johnstone, Mathew et al. 1991, Yang and Gould 2013). Misfolded soluble proteins were also found to be released in EVs (Bellingham, Guo et al. 2015). For example, during their maturation, reticulocytes release transferrin from their surface in EVs (Johnstone, Mathew et al. 1991), and oocytes release the sperm receptor juno4 in EVs right after fertilization (Bianchi, Doe et al. 2014). EVs may play a similar role in removing RNA, as it has been shown that a high ratio of miRNA to target mRNA induces the release of the miRNA in EVs (Squadrito, Baer et al. 2014). Several self-aggregating neurotoxic proteins can be released from cells in EVs, including Aβ (Rajendran, Honsho et al. 2006), APP C-terminal fragments (Perez-Gonzalez, Gauthier et al. 2012), Tau (Saman, Kim et al. 2012), α-synuclein (Emmanouilidou, Melachroinou et al. 2010), SOD1 (Grad, Yerbury et al. 2014) and the prion protein (PrP) (Fevrier, Vilette et al. 2004). Differences in the RNA profiles of EVs have also been reported in studies of neurodegenerative disease (Van Giau and An 2016). In addition to neurodegenerative disorders, EVs may play key roles in other pathological conditions. For example, EVs released from cancer cells regulate the tumor microenvironment (Milane, Singh et al. 2015), induce drug resistance and angiogenesis (Brinton, Sloane et al. 2015), and facilitate metastasis (Hoshino, Costa-Silva et al. 2015).

Accumulating evidence suggests that the release of EVs often serves as an alternative disposal pathway to the lysosome. In this review article we will summarize the evidence that lysosomal activity plays a role in the secretion of EVs and sorting of their cargo. We will also review what is known about the fate of EVs in the recipient cells and we will consider the possibility that they mediate transcellular autophagy.

Exosome biogenesis

Exosomes are released from cells when MVB, which are part of the endosomal – lysosomal system, fuse with the plasma membrane and release their ILVs (Colombo, Raposo et al. 2014). The endosomal system is well known for its role in the quality control and degradation of membrane proteins. Most membrane proteins are rapidly recycled between the plasma membrane and the early endosomal compartment. In the early endosome these proteins are either sorted to vesicles that proceed to late endosomes/MVB or to vesicles that recycle back to the plasma membrane (directly or through the recycling endosome compartment). The late endosomes/MVB fuse with lysosomes where their cargo is degraded, and some degradation products such as amino acids are made available for reuse by the cell (Huotari and Helenius 2011). However, MVB can also fuse with the plasma membrane, releasing their ILV cargo to the extracellular fluid as exosomes, and the MVB membrane is incorporated into the plasma membrane in a process known as backflow (Colombo, Raposo et al. 2014) (Figure 1). The process by which the fate of a protein is determined, whether recycling back to the plasma membrane, secretion in exosomes or sent to the lysosome for degradation is not fully established. However, at least part of this process is mediated by invagination of vesicles into the endosome lumen to form ILVs. Electron microscopy images show that while early endosomes contain few if any ILVs, late endosomes typically contain 3–20 ILV, and because of this morphology can also be termed MVB (Hanson and Cashikar 2012).

The main mechanism of ILV formation is mediated by the endosomal sorting complexes required for transport (ESCRT) machinery. The ESCRT machinery contains 4 protein complexes: the ESCRT-0 complex interacts with and attaches monoubiquitinated transmembrane proteins to the endosomal membrane; the ESCRT-I and -II complexes control the formation of membrane buds that contain transmembrane proteins as cargo; the ESCRT-III complex severs the connection to the endosomal membrane, which generates a separated ILV. The ESCRT machinery is released from the vesicle for another round of invagination by the AAA-ATPase VPS4 (Colombo, Raposo et al. 2014). In an unbiased screen Colombo et al. used siRNA to inhibit 23 different ESCRT proteins and measured exosome release by capturing them on beads with CD63 antibody (a common EV marker) and detection with CD81 and MHC-II antibodies. Interestingly, while knocking down HRS and stem1 (ESCRT-0) and TSG101 (ESCRT-I) significantly inhibited EV release, knocking down CHAMP4c (ESCRT-III), VPS4B, VTA1, and ALIX (disassembly complex) significantly increase EV secretion (Colombo, Moita et al. 2013). The influence of ALIX on EV secretion may be independent of its role in the ESCRT machinery as ALIX can also regulate EV secretion by interacting with syntenin and syndecan in response to heparin on the cell surface (Baietti, Zhang et al. 2012). Moreover the effect of ESCRT proteins may not be related to its endosomal roles, as it has been reported to have non-endosomal functions as well (Hurley 2015).

Exosome secretion and ILVs invagination are also regulated in an ESCRT independent manner that includes tetraspanin webs and lipids rafts (Yanez-Mo, Siljander et al. 2015). Tetraspanins are a family of proteins characterized by the presence of four hydrophobic transmembrane domains and conserved intracellular loops. CD63, CD9, CD81 and CD82

are members of this family that are abundant in EVs and commonly serve as markers for their purification (Andreu and Yanez-Mo 2014). Tetraspanins serve as a scaffold to anchor many proteins that participate in cell adhesion and signaling to a specific site on the membrane. While most tetraspanin proteins are predominantly present on the plasma membrane, CD63 is mostly present on the surface of endosomes. It has been reported that CD63 has a tendency to generate large protein domains on the inner surface of late endosomes that can change the physical properties of the membrane in a manner that encourages invagination (Piccin, Murphy et al. 2007, Bari, Guo et al. 2011). An additional mechanism for exosome biogenesis involves the accumulation of the bioactive lipid ceramide in the endosomal membrane (Trajkovic, Hsu et al. 2008). Ceramides form lipid patches (similar to lipid rafts) that interact with and accumulate proteins. The accumulation of many proteins and the tendency of these types of lipids to form vesicles promotes membrane invagination. Inhibition of ceramide synthesis by knocking down snMASE2 or chemical inhibition with GW4863 significantly reduces EV secretion (Trajkovic, Hsu et al. 2008, Yuyama, Sun et al. 2012). More recently it has been suggested that the effect of ceramide on EV secretion is mediated by local production of its downstream metabolite sphingosine-1-phosphate (S1P) (Kajimoto, Okada et al. 2013). Inhibition of S1P production reduces the levels of CD63, CD81 and flotillin (but not the EGF receptor) in ILVs (Kajimoto, Okada et al. 2013), suggesting that S1P plays a role in the sorting of ILVs into MVBs targeted for secretion of exosomes or for lysosomal degradation. It is unclear whether it is the ILV biogenesis pathway that determines if the vesicle will be sorted to the lysosome or to the plasma membrane for exosome release. It is also unknown if the sorting between these two fates is at the level of individual ILVs or the entire MVB.

It is important to note that MVBs can fuse with the plasma membrane, but may also form a kiss-and-run structure in which only some of the ILVs are released as exosomes (Colombo, Raposo et al. 2014). MVB intracellular trafficking is regulated by several Rab and SNARE proteins. The role of Rab proteins in EV secretion was tested in a shRNA screen of 59 different Rab protein members, the identification of EV secretion was performed by FACS analysis using beads coated with CD63 antibodies for capture and CD81,HLA_DR and Annexin V for detection (Ostrowski, Carmo et al. 2010). This unbiased screen showed that knockdown of Rab2b, Rab5a, Rab9a, Rab27a and Rab27b significantly decreased EV levels in the cell culture medium. The effect of Rab27 was further analyzed and was found to mediate the size density and intracellular localization of MVBs. In Rab27 knockdown cells, MVB density next to the plasma membrane was significantly reduced while MVB accumulation in the perinuclear region was increased. It has been suggested that Rab27 regulates the docking of MVB with the plasma membrane by interacting with the SNARE protein VAMP7 (Ostrowski, Carmo et al. 2010). In an independent screen to analyze the role of Rab proteins in EV secretion from oligodendrocytes in which Rab protein function was inhibited by overexpressing the Rab inhibitor family GAP (Rab GTPase-inactivation protein), the secretion of EVs was quantified by the presence of PLP-EGFP. It was found that inhibition of Rab5, Rab43, Rab7, Rab27a and Rab35 reduces the levels of PLP-EGFP in the cell culture medium, probably by reducing EV secretion. Further in-depth analysis shows that Rab35 also participates in the trafficking of MVBs to the plasma membrane and likely plays a role in their docking to the plasma membrane (Hsu, Morohashi et al. 2010).

Interestingly, even though it was not shown in the shRNA and GAP screens, Rab11 was shown to regulate EV secretion from erythrocytes (Savina, Vidal et al. 2002) and neurons (Beckett, Monier et al. 2013, Escudero, Lazo et al. 2014). In erythrocytes, Rab11 regulates the trafficking and docking of MVB to the plasma membrane, but not their fusion, which is regulated in a calcium-dependent manner by SNARE proteins such as VAMP7 (Savina, Furlan et al. 2003, Fader, Sanchez et al. 2009). There could be many reasons why different Rab proteins have been reported to regulate EV secretion including the cell type studied, the Rab inhibition method, and the marker used to measure EV secretion. However, all studies show that Rab proteins regulate MVB trafficking and docking to the plasma membrane, but not their fusion with the membrane (Figure 1).

Rab and SNARE proteins also regulate lysosome fusion with MVBs and autophagosomes (Szatmari and Sass 2014). The mechanism of the fusion is not completely characterized yet, but it has been shown to involve Rab7 and Rab11 and the SNARE proteins VAMP7, VAMP3, VAMP8, syntaxin 11, syntaxin 8, HOPS, NSF and vti1b (Huotari and Helenius 2011, Luzio, Hackmann et al. 2014, Szatmari, Kis et al. 2014). It is reasonable to assume that the SNARE proteins mostly participate in the fusion event, after the vesicle membranes are already closely apposed. What determines whether MVB dock with the plasma membrane or the lysosome (Figure 2)? Is this mechanism unique to MVB or is the same mechanism operative on autophagosome and/or recycling endosomes? As described below, answering these questions will be critical for understanding the role of EVs in protein homeostasis (proteostasis).

Roles of lysosomes in regulating exosome secretion

The mechanisms that sort MVB to the plasma membrane and the lysosome are unclear, but the existence of a decision point between the two fates suggests that inhibition of one pathway will increase the other. In support of the latter hypothesis, it has been shown that inhibition of the lysosome with different alkaline agents increase EV secretion. Treating cells expressing α-synuclein with Bafilomycin A, a lysosome inhibitor, increases the levels of α-synuclein released in EVs (Alvarez-Erviti, Seow et al. 2011). Bafilomycin A treatment also increases EV secretion as measured by nanoparticle tracking analysis (NTA) and the levels of LAMP1 and LC3 associated with them (Miao, Li et al. 2015). We found that Bafilomycin A increases the secretion of Aβ associated with EVs (unpublished observation), and chloroquine increases the secretion of the β-amyloid precursor protein C-terminal intracellular domain (ACID) in EVs (Vingtdeux, Hamdane et al. 2007). Mutations in Tau that induce lysosome inhibition were also shown to increase the expression of proteins associated with EV secretion (Simon, Garcia-Garcia et al. 2012, Wren, Zhao et al. 2015). Similarly, mutations in VPS4 (VPS4 is required for MVB maturation and fusion with the lysosome) increased the levels of EV-associated α-synuclein (Hasegawa, Konno et al. 2011). Moreover, there are several pathways that regulate both lysosome function and EV secretion. Ceramide and its metabolite S1P induce autophagy and EV secretion (Kajimoto, Okada et al. 2013, Huang, Berdyshev et al. 2015). Several ESCRT proteins including ALIX, HRS and TSG101 have been found to be essential for autophagy flux and EV secretion (Rusten and Simonsen 2008, Colombo, Raposo et al. 2014). In *Caenorhabditis elegans* it has been found that the VHA5 subunit of the lysosomal V-ATPase ion pump regulates the secretion of EV-

associated Hedgehog proteins in a manner distinct from its lysosomal function (Liegeois, Benedetto et al. 2006). Cellular metabolic status may also regulate both lysosomal functionality and EVs secretion. Knockdown of GAIP interacting protein C-terminus (GIPC), part of G-protein coupled receptor complex, in pancreatic cancer cells inhibit the expression of the glucose transporter Glut1 and glucose uptake. This reduction in glucose uptake induced AMPK phosphorylation and reduced mTOR phosphorylation, which generate accumulation of LC3-II and increased EVs concentration in the media (Bhattacharya, Pal et al. 2014). The accumulation of LC3-II can be due to enhanced autophagy initiation or reduced degradation in the lysosome.

More direct evidence for the involvement of the autophagy-lysosome pathway in EV secretion was recently shown by Murrow et al. (2015) who showed that the ubiquitin-like ATG12 protein, and its ligase ATG3, regulate basal autophagy, but not starvation-induced autophagy. The latter actions of ATG12 and ATG3 are mediated by their interaction with ALIX which regulates fusion of LC3-positive autophagosomes with MVBs and the release of their ILVs as exosomes (Murrow, Malhotra et al. 2015). This shows that autophagy may be directly linked with exosome secretion (Figure 1). Moreover, even though further direct evidence is needed, it seems that inhibition of lysosomal function increases EV secretion. This suggests that a cell can regulate the trafficking of MVBs to either the lysosome or the plasma membrane (Baixauli, Lopez-Otin et al. 2014), and further suggests that EV release may compensate for lysosomal dysfunction or overload to dispose of misfolded proteins. An influence of lysosome function on EV biogenesis suggests a mechanism for the coordinated regulation of autophagy and EV release under physiological conditions.

EVs as an alternative disposal pathway

EVs provide a mechanism for paracrine and autocrine signaling through delivery of proteins, RNAs, and lipids (Yanez-Mo, Siljander et al. 2015). However, as mentioned above, EVs can also be a device to relieve proteotoxic stress. The MVB is at the intersection between lysosomes and plasma membrane, thus it is reasonable to suggest that when the lysosome pathway is busy, the MVB is rerouted to the plasma membrane. Under what conditions will cells expel their molecular “garbage” in EVs? Likely possibilities include under conditions of oxidative and metabolic stress, and when the lysosome- and proteasome-mediated degradation and recycling machineries are overloaded. Are EVs expelled in a non-specific manner, or can they be targeted to phagocytic cells? In the following sections we will review the evidence that EVs function in an alternative disposal mechanism in lysosomal storage diseases and neurodegenerative disorders, and in response to infection by pathogens attack and certain drugs (Figure 3).

Lysosomal storage diseases

Lysosomal storage diseases (LSDs) include approximately 50 different inherited diseases that are characterized by a buildup of partially or undigested cellular waste products in lysosomes and, in some disorders, the endosomal system (Chen, Li et al. 2010, Biffi 2015, Chen, Qiu et al. 2016). These diseases result from mutations in several different genes. As previously mentioned, it is likely that if the lysosomes are malfunctioning, materials found

in MVBs will be redirected into the extracellular space. As such, it is reasonable to hypothesize that the concentration and content of EVs will be specifically modified in LSDs. A prime example of this is Niemann-Pick Type C disease (NPC), in which there is an accumulation of cholesterol in the lysosomes and endosomes due to mutations that cause loss of *npc1* gene function (Strauss, Liu et al. 2002, Liscum 2007, Strauss, Goebel et al. 2010). In one study it was shown that the addition of free cholesterol, an inhibitor of cholesterol transport (U18666A) or knockdown of NPC1 in oligodendroglial cells led to an accumulation of cholesterol in the endosomes (Strauss, Goebel et al. 2010). These treatments also induced the secretion of cholesterol in EVs. As further confirmation of the role of *npc1*, the up-regulation of EVs release in response to cholesterol was reversed by the expression of wild-type *npc1*. This suggests that EVs can be used as a method for eliminating accumulating intracellular cholesterol. Importantly, there is thought to be a tie between the lipid environment and the creation of vesicles within MVBs (Denzer, Kleijmeer et al. 2000, Fevrier and Raposo 2004) further implying an alteration in overall exosome formation and release in NPC1. Indeed, NPC1, a protein involved in lipid transport has been found not only in endosomes but in EVs as well (Liscum 2007). It is hypothesized that NPC1 may be responsible for detecting elevated levels of cholesterol and inducing its secretion by EVs (Liscum 2007); in this way, EVs contribute to the maintenance of cholesterol homeostasis. Whether this role of EVs is specific to NPC or takes place in other LSD is unclear.

It is likely that the ability of EVs to compensate for lysosome overload or dysfunction depends on the specific genetic aberrancy. Mutations that induce lysosome dysfunction directly, but leave the endocytic pathway unharmed, would be expected to increase EV secretion, while mutations that inhibit the entire endosomal-lysosomal pathway would be expected to reduce EV secretion. For example, Chediak-Higashi syndrome (CHS) is a rare LSD in which mutations in a single gene (*lyst*) lead to dramatically enlarged lysosomes. Intriguingly, it was found that although MVBs maintained normal morphology and size in CHS cells, there were decreased lysosomal markers commonly found in MVBs including LAMP1 and CD-63 (Faigle, Raposo et al. 1998). Instead, there was an increase of these markers on the plasma membrane, indicating an issue in transport from the golgi or early endosomes to MVB. Thus, it is predicted that exosome synthesis is inhibited and not able to compensate for the lysosomal dysfunction in CHS (Faigle, Raposo et al. 1998, Huizing, Anikster et al. 2001, Huizing and Gahl 2002). In this circumstance, microvesicle blebbing from the plasma membrane and release of aggregated membrane proteins may serve as the main method of compensation for the malfunction pf endosomal-lysosomal system.

Future work examining alterations of the endocytic-lysosomal system in LSD will lend insight to the consistency of these findings between disorders. If it is consistently seen that EV generation is upregulated in LSD and that their molecular content is altered based on the mutation, it is possible that EVs in biological fluids may serve as biomarkers of disease progression and efficacy of potential therapeutic interventions.

Viruses and other pathogens

Several viruses and intracellular bacteria enter the cells through the endocytic pathway (Nour and Modis 2014). Moreover, autophagy plays a role in the cellular immune system by engulfing intracellular pathogens and delivering them for degradation in the lysosomes (Steele, Brunton et al. 2015). MVBs and lysosomes transport cargo from the endocytic and autophagy pathways and thus are major subcellular locations of invading pathogens. Many of these pathogens evolve ways to avoid degradation, by escaping from the endosome, inhibiting MVB-lysosome fusion, or modifying lysosome pH (Nour and Modis 2014). Numerous studies have shown that the infection of cells with viruses or bacteria has a large effect on the cargo secreted by EVs (Hosseini, Fooladi et al. 2013). Moreover EVs released from infected cells contain proteins of the pathogen and in the case of virus, even their genome (Nour and Modis 2014, Schwab, Meyering et al. 2015). The release of EVs was reported to have various effects on the host-pathogen interaction (Hosseini, Fooladi et al. 2013, Sampey, Saifuddin et al. 2015). The intracellular mechanism by which the pathogen alters the EV cargo is less understood, and in all probability different pathogens use different mechanisms. Recently Miao et al. (2015) reported that viable cells can release large (1 μM) vesicles containing *E. coli*. These bacteria-containing vesicles were found in urine samples and were able to protect the bacteria from a membrane impermeable antibiotic (gentamycin). Interestingly these bacteria-containing vesicles exhibited many exosomal markers including CD63, ALIX and TSG101, but also the autophagy markers LC3, beclin1 and ATG5. Electron microscopy images show that bacteria-containing vesicles are found inside autophagosomes, MVBs and lysosomes. It was also shown that their release from infected cells is regulated by ALIX and TSG101, both of which also regulate EV secretion. A trigger for their secretion was inhibition of the lysosomal cation channel TRPML3, which normalized the lysosomal pH and induced the release of calcium. This facilitated the fusion of the lysosome with the plasma membrane and thus the release of its ILVs, including exosome-like vesicles and the large bacteria-containing vesicles (Miao, Li et al. 2015). The reliance of the EV bacteria secretion on neutralization of the lysosome pH was also shown by the lack of secretion of other *E. coli* strains which do not possess the ability to inhibit TRPML3, and thus neutralize lysosome pH. However, adding Bafilomycin A, a drug that neutralizes the lysosomal pH, to these cells induced the release of the bacteria in EVs (Miao, Li et al. 2015). This example shows that when cells cannot degrade the bacteria due to the neutralization of the lysosomal pH, they instead extrude the bacteria in EVs.

Several viruses hijack the endosomal system in order to enter cells and thereby avoid recognition by the immune system components (Gould, Booth et al. 2003). Some viruses even utilize EVs to spread among cells. Hepatitis C virus (HCV) full-length viral RNA along with all of its core and envelope proteins were found in EVs released from infected cells, and such EV release is regulated by the ESCRT protein HRS (Tamai, Shiina et al. 2012, Ramakrishnaiah, Thumann et al. 2013). These virus-containing EVs are highly efficient in infecting naïve cells. Interestingly the packaging of HCV in EVs is mediated by autophagy (Shrivastava, Devhare et al. 2015) and thus may be similar to the release of intracellular bacteria as described above. Hepatitis A is another non-enveloped virus that utilizes EVs for infecting other cells (Nour and Modis 2014). Cells infected with the experimental virus

adeno-associated virus (AAV) release it within EVs. Interestingly AAV particles in EVs are more infective than free AAV particles (Maguire, Balaj et al. 2012). However, not all viruses use EVs for infection; for example, even though HIV full-length RNA was found in EVs they lack the ability to infect other cells (Nour and Modis 2014). However HIV, herpes virus, Epstein–Barr virus as well as the intracellular mycobacterium secrete their proteins via EVs (Hosseini, Fooladi et al. 2013). Whether the extrusion of viral nucleic acids and protein in EVs is because the cell actively packages these foreign molecules to protect itself, and/or is a mechanism whereby pathogens manipulate the host to facilitate its spread is not clear. It is also not clear if the secretion is due active sorting or just due to high levels of virus inside the cells.

Neurodegenerative disorders

Many neurodegenerative diseases including prion diseases, Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) involve the abnormal accumulation of self-aggregating proteins within, or in some cases outside of neurons (Walker et al., 2006). In AD, Tau protein aggregates accumulate in the cytoplasm and amyloid β-peptide (Aβ) accumulates both extracellularly and intracellularly (Ditaranto et al., 2001). α-synculein and PrPsc accumulate in the neuronal soma in Parkinson's disease and prion disease, respectively, while in HD, mutant huntingtin protein forms intranuclear inclusions. In these neurodegenerative diseases as well as others, the autophagy-lysosomal pathway is impaired and unable to overcome the pathological protein load (Nixon 2013). Interestingly many of these proteins were reported to be released in EVs (Bellingham, Guo et al. 2015).

PrPsc was found to be present in EVs using numerous methods, including immunoblot, light microscopy, electron microscopy and mass spectrometry analyses (Fevrier, Vilette et al. 2004, Robertson, Booth et al. 2006). Furthermore, EVs containing PrPsc are more infectious than is free PrPsc (Veith, Plattner et al. 2009) and transgenic mice inoculated with PrPsc-containing EVs subsequently develop prion disease (Fevrier, Vilette et al. 2004). Mutant PrPsc can increase autophagy flux and accumulate in the lysosomal compartment (Shin, Oh et al. 2013, Yao, Zhao et al. 2013). Thus, in this case the secretion of PrPsc in EVs is likely not due to lysosome inhibition, but may be a consequence of an overload of the autophagy pathway.

Accumulations Aβ and Tau are a major hallmarks of AD (Mattson 1995). Both of these proteins were found in EVs released from neural cells in different AD cell culture models, and in EVs isolated from CSF, brain extracellular space, and blood samples from AD patients (Rajendran, Honsho et al. 2006, Perez-Gonzalez, Gauthier et al. 2012, Saman, Kim et al. 2012). Tau is a microtubule-associated protein in axons of neurons, and its hyperphosphorylation (p-Tau) results in microtubule depolymerization and the formation of toxic p-Tau aggregates. Accumulations of Aβ and p-Tau, as well as mutations in presenilin1 (PS1) that cause early-onset inherited AD, inhibit autophagy flux and lysosomal function (Nixon 2013). The release of these pathological proteins in EVs may compensate for the reduction in their degradation inside the cells. In support of this notion, preventing membrane trafficking from endosomes to lysosomes by treatment of cells with chloroquine

increases the amount of Aβ secreted in association with EVs (Yuyama, Yamamoto et al. 2008, Yuyama, Sun et al. 2012), and incubating PS1 mutant-expressing H4 cells with Bafilomycin A has a similar effect (our unpublished observation). It is also possible that this secretion may be a mechanism to prevent disruption of lysosomal membrane permeability, as Ditaranto et. al hypothesize that the intracellular accumulation of soluble Aβ42 within the late endosome and lysosome may lead to the initiation of cell death through the leakage of lysosome contents (2001). EVs are not only releasing Aβ, but also interact with it extracellularly, via binding of Aβ to proteins and lipids on the EV surface, and facilitate its uptake by microglia (Yuyama, Sun et al. 2012). This suggests that beyond having a role in clearing intracellular misfolded and aggregated proteins, EVs may interact with pathogenic proteins in the extracellular environment and facilitate their clearance by phagocytic cells.

An additional example of increased EVs secretion under conditions of lysosomal dysfunction comes from studies of frontotemporal dementia with Parkinsonism related to chromosome 17 (FTDP-17) caused by the Tau N279K mutation. The levels of the EV marker flotillin-1 were significantly increased in frontal and temporal cortices of PPND/FTDP-17 patients with the N279K Tau mutation. NSCs derived from patients with the N279K Tau mutation displayed impaired endocytic trafficking as evidenced by accumulation of endosomes, reduction of lysosome numbers, and an increase in common EV markers such as flotillin1 (Wren et al., 2015).

Emerging findings suggest the role of lysosomal dysfunction and EVs secretion in the pathogenesis of synucleinopathies, including PD, dementia with Lewy bodies, and multi-system atrophy. Gaucher disease (GD), a lysosomal storage disorder, has been clinically linked to PD. Functional loss of GD-linked gluocerebrosidase (GCase) in mice primary neurons or human patient-derived iPS cells differentiated into neurons compromises lysosomal protein degradation and contributes to α-synuclein aggregation. In addition, α-synuclein accumulation inhibits the lysosomal activity of normal GCase in neurons and idiopathic PD brain, thus forming a positive feedback loop (Mazzulli, Xu et al. 2011). The effect of GCase activity on EV secretion has not yet been examined, but inhibition of lysosomal activity with Bafilomycin A1 increased the secretion of α-synuclein oligomers in EVs from SNCA-expressing H4 cells and SH-SY5Y neurons and Inhibition of the lysosome with ammonium chloride had a similar effect (Alvarez-Erviti, Seow et al. 2011). Systemic administration of Bafilomycin A1 results in markedly increased α-synuclein levels in the CSF of wild-type and α-synuclein transgenic mice (Poehler, Xiang et al. 2014). The secretion of α-synuclein not only depends on the lysosome but also on calcium (Emmanouilidou, Melachroinou et al. 2010), which may be linked to lysosomal function by pH-sensitive calcium channels in the lysosomal membrane. EV-associated α-synuclein is present on both the outside and inside of EVs and is more likely to be taken up by recipient cells and capable of inducing more toxicity compared to free α-synuclein oligomers (Danzer, Kranich et al. 2012). Moreover, inhibition of the lysosome with Bafilomycin A further exacerbates EV-associated α-synuclein cytotoxic effects including neuronal uptake and activation of pro-inflammatory microglia (Alvarez-Erviti, Seow et al. 2011).

Loss-of-function mutations in PARK9 cause juvenile-onset PD. Two recent studies found that PARK9 (ATP13A2) is located on MVBs in the human H4 and HEK 293T cells, and

primary neurons where it regulates ILV formation and exosome secretion. PARK9 mutations reduce EV secretion from human H4 cells and mouse N2a cells, while PARK9 overexpression increases their secretion. PARK9 may promote zinc accumulation in MVBs and may also regulate ESCRT protein activities. Interestingly, PARK9 overexpression increases the levels of EV-associated α-synuclein by 3 fold and its inhibition significantly reduces the secretion of α-synuclein in EVs. These results suggest that PARK9 directly regulates the clearance of α-synuclein in exosomes, but it is also possible that the effect is mediated by inhibition of the lysosome. Interestingly, upregulation of PARK9 expression is found in surviving dopaminergic neurons in sporadic PD patients, suggesting that its effect on α-synuclein secretion in EVs protects cells from the PD pathology (Kong, Chan et al. 2014, Tsunemi, Hamada et al. 2014). If the expression of PARK9 in the surviving dopaminergic neurons protects them by increasing EV secretion, it would be interesting to examine whether the expression of PARK9 also promotes degeneration of neighboring neurons via transfer of EVs containing α-synuclein.

An additional link between PD and EV secretion was shown in a recent report demonstrating that PD patients have reduced levels of VPS4 in neurons with Lewy bodies. Dominant-negative mutant VPS4 impairs biogenesis of MVBs, thereby interfering with lysosomal targeting of α-synuclein and facilitating its secretion into the extracellular milieu. Intriguingly, functional disruption of the recycling endosome regulator Rab 11a efficiently restores the hypersecretion of α-synuclein in VPS4-defective HEK 293T and SH-SY5Y neuronal cells (Hasegawa, Konno et al. 2011).

Because EVs dispose of pathogenic proteins, EVs isolated from biological fluids have considerable potential for the development of biomarkers for disease diagnosis and treatment evaluation. The levels of the AD-related proteins phospho (P)-serine-type 1 insulin receptor substrate (IRS-1), P-S396-Tau, p-T181-tau, and Aβ1–42 were significantly higher in neuronal-derived blood EVs from diagnosed AD patients, and, in preclinical AD subjects, even 10 years before clinical disease onset compared to age-matched control subjects (Fiandaca, Kapogiannis et al. 2015, Kapogiannis, Boxer et al. 2015). In addition, levels of cathepsin D, lysosome-associated membrane protein 1 (LAMP-1) and ubiquitinylated proteins were significantly higher, and mean levels of heat-shock protein 70 significantly lower, in plasma EVs from AD patients compared to age-matched control subjects in cross-sectional studies (Fiandaca, Kapogiannis et al. 2015), suggesting that an increased fraction of these EVs were of lysosomal origin in AD. The latter findings may lead to the development of novel EV-based biomarkers for early diagnosis of AD. Several PD-related proteins are detected in EVs isolated from biological fluids of PD patients. α-synuclein was detected in neuron-derived EVs isolated from plasma (Shi et al., 2014). The level of plasma EV-associated α-synuclein was significantly higher in PD patients, which suggests an increased efflux of the protein to the peripheral blood of these patients. In addition, a significant correlation is found between the level of plasma EV-associated α-synuclein and disease severity (Shi, Liu et al. 2014). Two additional proteins that are linked to PD, DJ-1 and LRRK2 are also detected in EVs isolated from urine samples from PD patients, and the level of DJ-1 is significantly higher in patients compared to control subjects (Ho, Yi et al. 2014). Furthermore, levels of syntenin 1, which is a regulator of EV biogenesis, are greater in EVs isolated from blood of PD patients (Tomlinson, Zheng et al. 2015). The discovery of

differences in disease-related proteins in EVs isolated from biological fluids of patients and control subjects might lead to early diagnosis and disease prognosis of neurodegenerative disorders. Moreover the existence of Aβ, p-Tau and α-synuclein in neuron-enriched EVs isolated from blood samples suggests that such EVs have a role in clearing these toxic proteins from the central nervous system, but this hypothesis needs further empirical evidence.

Drugs

Cells have the ability to antagonize drug effects by various mechanisms including metabolizing to an inactive form or expelling the drug from the cell. Exosomes have emerged as an additional mechanism for cancer cells to rid themselves of chemotherapy drugs (Azmi, Bao et al. 2013). For example, when lymphoma cells are treated with doxorubicin or pixantrone, which inhibit DNA synthesis and generate DNA damage, these drugs are found in high concentrations in EVs (Koch, Aung et al. 2015). In cells that develop resistance to these drugs, EV drug levels are elevated. The release of these drugs depends on the drug transporter ABCA3 located in the membrane of MVB. Interestingly, treatment with the ABCA3 inhibitor Indomethacin, which also inhibits V-ATPase activity, decreased the secretion of doxorubicin and pixantrone in EVs and significantly increased the potency of the chemotherapeutic drugs in vitro and in vivo (Koch, Aung et al. 2015). The reduction in EV-drug secretion in response to lysosome inhibition suggests that this EV-dependent mechanism of drug disposal is independent of the lysosome. Cisplatin, a drug that induces DNA crosslinking, has similarly been found in EVs (Safaei, Larson et al. 2005, Federici, Petrucci et al. 2014). Cisplatin resistant ovarian carcinoma cells release significantly more cisplatin in EVs. Interestingly, cisplatin-resistant cells has significantly less lysosome and LAMP1 expression, but higher levels of LAMP1 on their secreted EVs (Safaei, Larson et al. 2005). Thus, it is reasonable to speculate that the release of cisplatin in EV is regulated by the reduction in lysosome mass. However, because cisplatin is metabolized in the low pH environment of the lysosome, decreased lysosome function and the extrusion of the drug in EVs may be an alternative mechanism for drug resistance. While these are the only examples of the secretion of chemotherapy drugs in EVs, there are many more examples in which EVs mediate chemotherapy resistance via ABC transporters like P-glycoprotein and delivering different miRNAs to cancer cells (Azmi, Bao et al. 2013).

The role of EVs in extruding foreign compounds from a cell is not limited to chemotherapeutic agents and cancer cells. For example, anthrax toxin has also been found in MVBs and EVs. Anthrax is composed of two components, the protective antigen (AP) that can integrate into the membrane and form a channel, and the lethal factor (LF) that exerts the toxic effect. It has been shown that AP generates a channel in the MVB membrane to enable back flow of LF into the cytoplasm. This accumulation of LF in ILVs protects it from degradation and generates slow release of LF to maintain the LF inhibition of MAPK phosphorylation up to 8 days after treatment. Interestingly, this slow release depends on pH, as Bafilomycin A induces fast MAPK recovery (7 hours), and on MVB formation as ESCRT (TSG101, ALIX and SNX3) knockdown cells exhibit accelerated MAPK recovery (8–16 hours). The accumulation of LF in ILVs also induces its release in EVs in a Rab11- and Rab35-, but not Rab27- dependent manner. These EVs can be internalized into naïve cells

and induce long-term MAPK inhibition, which indicates that the internalized EVs enter to the endocytic pathway (Abrami, Brandi et al. 2013). LF is not degraded by the lysosome but by the proteasome, and thus releasing it in EV may be an alternative strategy for LF removal.

EVs may have roles in disposing larger foreign substances as well. Marchesano et al. (2013) used citrate-gold (14 nm) conjugated to c-myc siRNA to study internalization and distribution of the gold particles inside cells of *hydra vulgaris*. They visualized the particles by electron microscopy at different time points following the particle addition. Interestingly, the particles were rapidly internalized into cells within 30 minutes following their addition, and after 24 hours were found mostly in lysosomes and MVBs, and in secreted EVs. 48 hours following the particle addition the particles were predominantly found in EVs that had been released into the culture medium (Marchesano, Hernandez et al. 2013). While this study did not use functional assays, and thus we do not know if there was an effect on lysosome function, it successfully demonstrated the EVs are used to dispose of intracellular foreign agents.

Exosome fates in recipient cells

EVs have been shown to be internalized by various types of cells, with mostly unknown levels of specificity. The recipient cells may internalize EVs by different mechanisms including phagocytosis, endocytosis, pinocytosis and fusion with the plasma membrane (Mulcahy, Pink et al. 2014). Tian et al. labeled EVs with different membrane dyes and followed their internalization into PC12 cells by live imaging (Tian, Wang et al. 2010, Tian, Zhu et al. 2013). They found that EVs moved with different kinetics in different steps of the internalization process. First the EVs attached to the plasma membrane and slowly moved in a seemingly random manner. Then they were rapidly internalized into the cell, in a process that is probably mediated at least in part by actin filaments. Inside the cells, the EVs briefly moved freely (with no obvious directionality) and then the majority of them traveled toward the perinuclear region in a microtubule-dependent manner where they mostly fused with lysosomes (Tian, Zhu et al. 2013). The EV protein cargo was also labeled with TAMRA-NHS, which interacts with the free amino groups of proteins, and was tracked inside the cells. Upon interaction with lysosomes the membrane dye of the labeled EVs disappeared from the lysosome, probably due to lipid recycling back to the plasma membrane, whereas the EV protein cargo remained in the lysosome (Tian, Wang et al. 2010). The co-localization of around 60% of the internalized EVs with the recipient cell lysosome was also shown in cells treated with labeled EVs that had been isolated from fetal bovine serum (FBS). This was observed for both mice and human cell lines, suggesting that the lysosome co-localization may be due the foreign nature of the FBS EVs (Eitan, Zhang et al. 2015). The interaction of EVs with the lysosome does not necessarily imply that they are targeted for degradation; it could also mediate some of the biological effects of EVs on the recipient cell. For example, macrophage-derived EVs were shown to enter endothelial cell lysosomes and inhibit integrin trafficking and as a consequence cell migration (Lee, Kim et al. 2014). In a similar manner EVs released from SW480 colorectal cancer cells were found to be co-localized with HepG2 hepatocellular lysosomes following their incubation and inhibit their migration (Chiba, Watanabe et al. 2016). However, it is important to note that all of these

studies have been performed using membrane dyes for visualizing EVs. This approach has several limitations including that low levels of leakage of the dye from the membrane, which can lead to false-positive results; these dyes are very long-lived and thus staining can prevail long after the EVs been degraded. One study shows the interaction of EVs miRNA cargo with the recipient cells endo-lysosomal pathway without the use of a membrane-dye. In this case EVs released by lung cancer (Fabbri, Paone et al. 2012) and neuroblastoma (Challagundla, Wise et al. 2015) cells activate monocyte NFκb pathway and cytokines secretion, which induce tumor growth and drug resistance in-vitro and in-vivo. This effect is mediated by EV internalization into the monocytes endo-lysosomal system and interaction of mir21 and mir29 but not mir16 all found within the secreted EVs with TLR7/8. The interaction of the exogenous miRNA was shown by connecting the CY5 fluorophore to the miRNA released from the cancer cells and by transfecting them with the EV marker CD9 conjugated with GFP (Fabbri, Paone et al. 2012). While these results strongly suggest that the lysosome is a target of internalized EVs, more studies with a more specific staining for lipids, proteins or RNA would be required to determine the extent and mechanism of such interaction. In that regard, chimeric proteins targeted to EVs have been developed (Shen, Wu et al. 2011) and can be used alone or in combination with a lysosome protein in FRET analysis. Novel systems for tracking RNA loading and trafficking in EVs are also under development.

The trafficking of EVs between a donor cell and a recipient cell lysosome suggests a transcellular degradation mechanism of physiological relevance. To date there are only a few examples of transcellular autophagy, and EVs are only one suggested mechanism, with the formation of intercellular nanotubes being another mechanism (Kaushik and Cuervo 2015). The reason that only a few examples of transcellular autophagy have been reported thus far may be a consequence of methodology limitations, because it requires a clear demonstration of transfer of a protein or organelle from one cell to another and their lysosomal fate in the recipient cell. This has been demonstrated for transmitophagy, in which dysfunctional mitochondria in optic nerve axons are transferred to adjacent astrocytes (Davis et al., 2014). The authors developed a novel construct that contains cytochrome c-mCherry-GFP, which enabled them to follow the protein delivery to the lysosome (low pH environment) where the GFP fluorescence is quenched but the mCherry fluorescence is preserved. Thus, cytochrome c-associated fluorescence will appear yellow in the mitochondria and cytoplasm and red in the lysosome. It was shown that the mitochondrial protein was delivered from optic nerve axons into astrocytes, wherein it interacted with the lysosome. Further analysis showed that the transfer was mediated by the packaging of mitochondria in large EVs that were internalized by astrocytes and fused with their lysosomes (Davis, Kim et al. 2014). It remains to be determined whether this mechanism is specific for the optic nerve or if it takes place in other regions of the nervous system and other tissues.

The autophagy-lysosome pathway is not only important for the disposal of damaged/aggregated proteins and dysfunctional organelles, but also for nutrient recycling. The role of EV interaction with the recipient cell lysosome in nutrient transfer has not been directly investigated. However, it has been reported that culturing cancer cells with medium containing EV-depleted FBS reduces their proliferation and migration compared to cells with medium containing FBS containing EVs (Ochieng, Pratap et al. 2009, Shelke, Lasser et

al. 2014, Eitan, Zhang et al. 2015). We found that HEK cells grown in EV-depleted medium have elevated expression of genes related to lipid biogenesis, mostly sterols (unpublished observations), which may be induced to compensate for the lack of the FBS EV source of such lipids. Moreover EVs may deliver lysosomal proteins between cells. For example EVs derived from mesenchymal stem cells can deliver WT CTNS to the lysosomes of CTNS mutant fibroblasts, which significantly increases cysteine levels in recipient mutant cells (Iglesias, El-Kares et al. 2012). EVs were also reported to deliver essential proteins from the epididymis to mature spermatozoa, presumably because spermatozoa do not express those proteins (Sullivan, Saez et al. 2005).

Altogether, it seems that the lysosome is a major target for internalized EVs. This suggests a role of EVs in systemic protein homeostasis. However, more direct evidence is needed to understand the degree of such processes and how it contributes to different physiological and pathological conditions. In addition, it is unknown if there is a molecular signal that targets EVs to the lysosome and whether recipient cells recognize EVs as foreign elements.

Conclusions

It has become clear that cells can either target damaged molecules, aggregated proteins and foreign agents (viruses, bacteria, etc.) to the lysosomes OR they can package such potentially toxic entities in vesicles that are extruded from the cell. Studies of experimental models relevant to neurodegenerative disorders have shown that pathogenic self-aggregating proteins such as Aβ, p-Tau and α-synuclein can either be degraded in the autophagy-lysosome pathway or released in EVs. EVs released from one cell can be internalized by neighboring cells and targeted to the lysosomes of the recipient cell. Emerging evidence also suggests that EVs may mediate the transcellular spread of pathogenic proteins in disorders such as AD, PD and prion diseases. EVs extruded from cells of most organ systems are present in the blood and other biological fluids. Recent advances in methods for isolating cell type-specific EVs from the total pool of circulating EVs are leading to the development of EV-based biomarkers for the early diagnosis of diseases ranging from cancers to AD and PD. The molecular mechanisms that determine whether MVBs traffic to the lysosomes or to the plasma membrane are poorly understood, and identifying such mechanisms may lead to novel approaches for treating disorders that involve excessive accumulation of pathogenic proteins or lipids.

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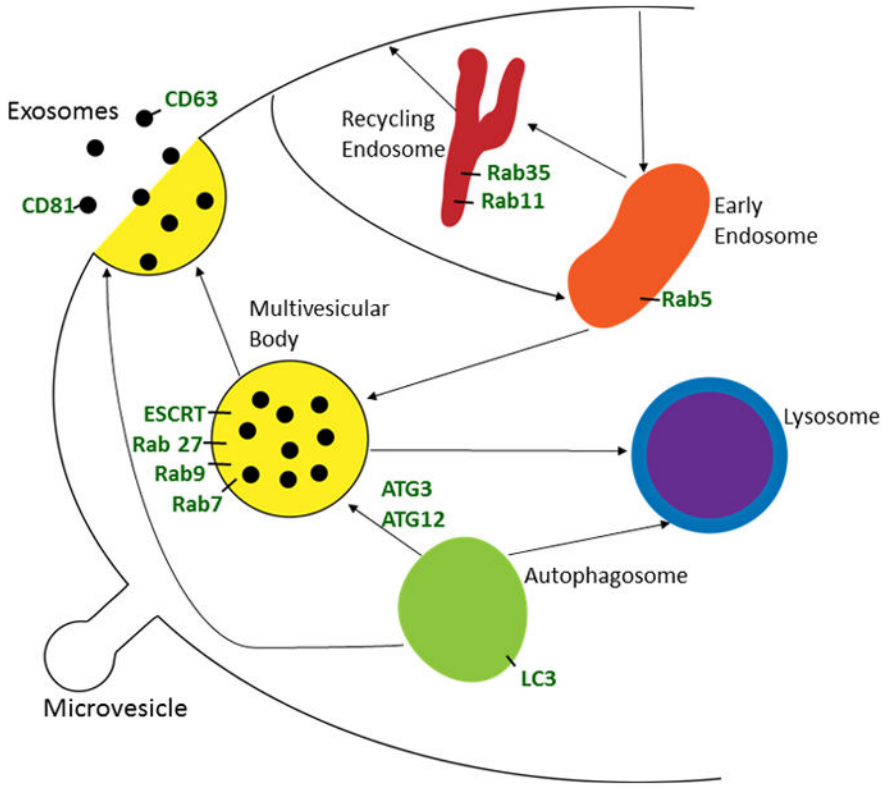

Figure 1.

Subcellular pathways for the packaging, lysosomal degradation and vesicular extrusion of damaged and aggregated proteins, or foreign agents (viruses, bacteria, etc.).

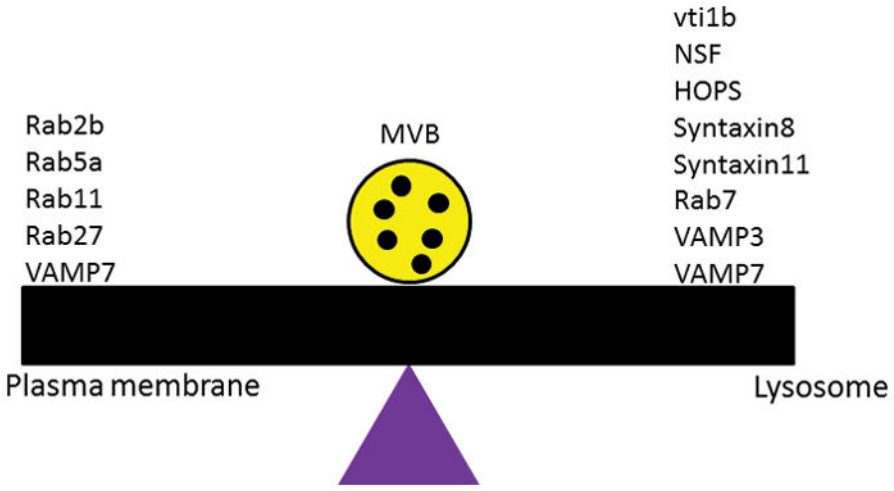

Figure 2.
Examples of proteins that may target MVBs to either the lysosome or the plasma membrane.

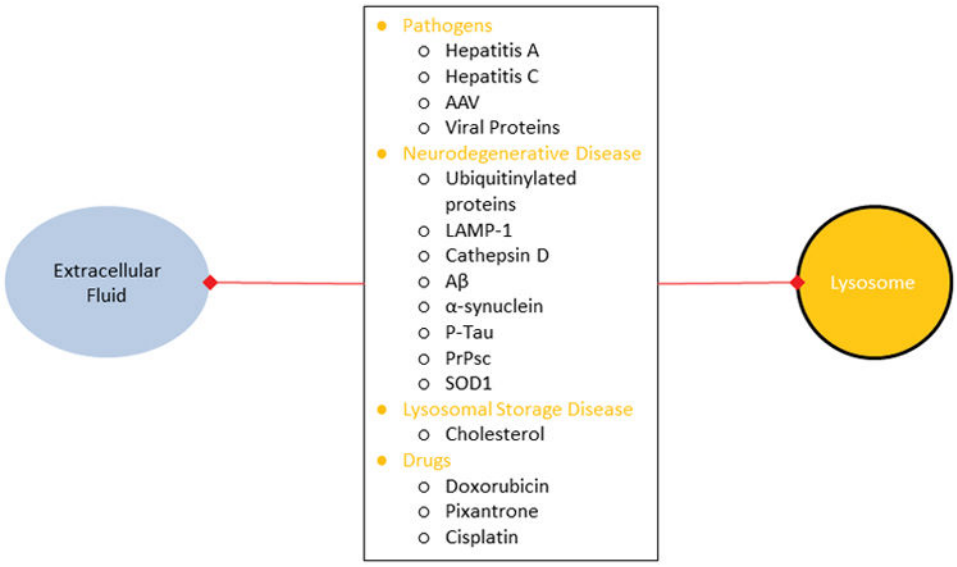

Figure 3.

Examples of pathogens, neurodegenerative disorder-related proteins, lipids and drugs that can be disposed of by lysosomal degradation or extrusion in EVs.