Exosomes mediate intercellular communication and participate in many cell processes such as cancer progression, immune activation or evasion, and the spread of infection. Exosomes are small vesicles secreted to the extracellular environment through the release of intraluminal vesicles contained in multivesicular bodies (MVBs) upon the fusion of these MVBs with the plasma membrane. The composition of exosomes is not random, suggesting that the incorporation of cargo into them is a regulated process. However, the mechanisms that control the sorting of protein cargo into exosomes are currently elusive. Here, we review the post-translational modifications detected in exosomal proteins, and discuss their possible role in their specific sorting into exosomes.

Keywords: post-translational modifications, exosomes, ubiquitination, sorting, multivesicular bodies
Table 1 | Post-translational modification of eukaryotic proteins

<table>
<thead>
<tr>
<th>Groups of PTMs</th>
<th>Modification</th>
<th>Added group</th>
<th>Modified residues of target proteins</th>
<th>PTM in exosomal proteins</th>
<th>Biological relevance conferred by the PTMs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphorylation</td>
<td>Phosphate</td>
<td>Tyr, Thr, Ser, His</td>
<td>FasL; AnnexinA2; tau; γ-synuclein</td>
<td>Sorting into exosomes; incorporation into exosomal membrane; spreading of toxic aggregates through exosomes</td>
<td>(8–11)</td>
</tr>
<tr>
<td></td>
<td>Acetylation</td>
<td>Acetyl group</td>
<td>Lys</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Methylation</td>
<td>Methyl group</td>
<td>Lys, Arg</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Oxidation</td>
<td>Different</td>
<td>All amino acids, but preferentially Tyr, Phe, Trp, His, Met, Cys</td>
<td>γ-Synuclein</td>
<td>Spreading of toxic aggregates through exosomes</td>
<td>(11)</td>
</tr>
<tr>
<td></td>
<td>Nitrosylation</td>
<td>Nitric oxide</td>
<td>Cys, Met</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Addition of carbohydrates or glycosylation</td>
<td>N-linked glycosylation</td>
<td>Glycosyl group</td>
<td>Asn, Arg, and N-terminus Ser, Thr, and amino acids in close proximity to Tyr phosphorylation sites</td>
<td>Several glycoproteins</td>
<td>Sorting of particular glycoproteins into exosomes (?)</td>
<td>(12–14)</td>
</tr>
<tr>
<td></td>
<td>O-linked glycosylation</td>
<td>Glycosyl group</td>
<td>Ser, Thr, and amino acids in close proximity to Tyr phosphorylation sites</td>
<td>Several glycoproteins</td>
<td>Sorting of particular glycoproteins into exosomes (?)</td>
<td>(12–14)</td>
</tr>
<tr>
<td></td>
<td>C-linkedmannosylation and glycopilation of glycosyl-phosphatidylinositol (GPI)</td>
<td>Glycosyl-phosphatidylinositol (GPI) group</td>
<td>Carbon on a tryptophan side-chain and C-terminus, respectively</td>
<td>Several glycoproteins</td>
<td>Sorting of particular glycoproteins into exosomes (?)</td>
<td>(12, 13)</td>
</tr>
<tr>
<td>Addition of lipids (lipidation)</td>
<td>Palmitoylation</td>
<td>Palmitic acid</td>
<td>Cys</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>N-myristoylation</td>
<td>Myristoyl</td>
<td>N-terminal glycine residue</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Addition of amino acids</td>
<td>Glutamic acid</td>
<td>Gly</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

(Continued)
<table>
<thead>
<tr>
<th>Groups of PTMs</th>
<th>Modification</th>
<th>Added group</th>
<th>Modified residues of target proteins</th>
<th>PTM in exosomal proteins</th>
<th>Biological relevance conferred by the PTMs</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addition of other polypeptides</td>
<td>Ubiquitination Ubiquitin protein</td>
<td>Lys, N-terminus, non-lysine residues (Cys, Thr, Ser)</td>
<td>Several proteins, some examples: LMP2A; PTEN; SIMPLE; HSP70; ARRDC1</td>
<td>Exosomal LMP2A loading; Sorting of PTEN into exosomes; Unknown function; Unknown function; Secretion into shedding vesicles</td>
<td>(16–20)</td>
<td></td>
</tr>
<tr>
<td>SUMOylation (Small ubiquitin-related modifier addition)</td>
<td>SUMO1-4 proteins Tetrapeptide consensus motif Ψ-K-x-D/E (Ψ: hydrophobic residue, K: lysine conjugated to SUMO, x: any amino acid, D or E: acidic residue)</td>
<td>hnRNPA2B1</td>
<td>Regulate the binding of miRNAs to hnRNPA2B1</td>
<td>(21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEDDylation (Neural precursor cell-expressed developmentally down-regulated 8 addition)</td>
<td>NEDD8 protein Lys</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ISGylation (Interferon-stimulated gene 15 addition)</td>
<td>ISG15 protein Lys</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Isoprenylation</td>
<td>Farnesylation Farnesyl group Cys and sequence motifs CAAX, CC, or CAC at C-terminus (C: Cys, A: alanine, X: any amino acid)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Geranylgeranylation</td>
<td>Geranylgeranyl isoprene unit Cys and sequence motifs CAAX, CC or CAC at C-terminus (C: Cys, A: alanine, X: any amino acid)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>
The Epstein-Barr virus (EBV) protein LMP2A (latent membrane protein 2A) is ubiquitinated in exosomal fractions (16). LMP2A contains two PXYY motifs, through which it associates with neural precursor cell expressed developmentally downregulated protein 4 (Nedd4)-family ubiquitin ligases (16). Ubiquitination of LMP2A leads to endocytic transport of the protein from the plasma membrane to MVBs. Nedd4 E3 ubiquitin ligases are able to bind directly to target proteins though the PPXY motif, but proteins lacking this motif can bind Nedd4 through the adaptor Nedd4-family-interacting protein 1 (Ndfip1), leading to their ubiquitination (46). Ndfip1 overexpression increases the protein content of exosomes and enhances exosomal sorting of normally absent proteins, such as Nedd4, Nedd4-2, and Itch. These exosomal proteins moreover cargo [reviewed in Raiborg and Stenmark (39)]. The ESCRT complex consists of four subcomplexes, ESCRT-0, -I, -II, and -III, and several accessory proteins. ESCRT-0, -I, and -II contain ubiquitin-binding subunits that interact directly with ubiquitinated cargo. The directional flow of cargo from ESCRT-0 to ESCRT-I and -II might be regulated by PTMs. In fact, the ESCRT-0 subunits are known to be phosphorylated and to be mono-ubiquitinated (40–42). The latter modification keeps these subunits in an inactive form owing to intramolecular interactions between their ubiquitin interacting motifs and the appended ubiquitin (43, 44). However, the role of ubiquitin and the ESCRT complex in the sorting of proteins into ILVs for exosome secretion is still unclear, and MVB biogenesis, exosome secretion, and exosomal-protein sorting have been reported in an ESCRT/ubiquitin-independent manner [reviewed at Villarroya-Beltri et al. (45)].

FIGURE 1 | Post-translational modifications of exosomal proteins. Membrane receptors such as EGFR and MHCII are ubiquitinated and sorted to MVBs. Then, they follow a degradative pathway by the fusion with lysosomes. Note that non-ubiquitinated MHCII can be sorted into exosomes. Ubiquitinated LMP2A and ubiquitinated and phosphorylated FasL follow a secretory pathway where both modified proteins are delivered into exosomes. Non-membrane proteins like SUMOylated hnRNPA2B1, phosphorylated and oxidized γ-synuclein, and phosphorylated tau are packed into exosomes. Myristoylated TyA protein is able to oligomerize, leading to the formation of shedding vesicles. Ubiquitinated ARRD1 can induce plasma membrane budding by an ESCRT complex depending mechanism, producing ectosomes.
WW domain-containing protein 2 (WWP2). Down-regulation of ARRDC1 in vesicles is ubiquitinated by the E3 ligase upon interaction of the tumor susceptibility gene microvesicles (ARMMs). ARMMs directly bud from the plasma membrane. Moreover, directed-mutagenesis of WW-2 (MARCH) E3 ubiquitin ligase does not specifically lead these molecules into exosomes, and forcing MHC-II ubiquitination by expression of membrane-associated ring finger (C3HC4) 8 (MARCH) E3 ubiquitin ligase does not enrich MHC-II molecules in exosomes, though it does completely deplete them from the plasma membrane. Moreover, directed-mutagenesis of all MHC-II lysine residues does not impair the exosome sorting of these receptors. However, ubiquitination of the MHC-II cytoplasmic domain, required for recognition by the ESCRT complex, is important for sorting membrane MHC-II to MVBS for lysosomal degradation. The non-ubiquitination of MHC-II molecules present in exosomes suggests that this PTM is not involved in sorting to these vesicles. The two mechanisms for loading MHC-II into MVBS are engaged for different physiological functions. Thus, whereas ubiquitin-dependent sorting takes place in immature DCs, in which ubiquitinated receptors are degraded in lysosomes, in activated-DCs, non-ubiquitinated MHC-II-containing exosomes are efficiently delivered to interact with T cells, enhancing antigen specific MHC-II-mediated presentation. Heat shock protein 70 (HSP70) also seems to be sorted into exosomes independently of its ubiquitination. Thus, although deletion of the deubiquitin domain of COP9 signalosome complex subunit 5 (CSN5) enhances packing of ubiquitinated HSP70 into exosomes, knockdown of the entire CSN5 protein increases the levels of both modified and non-modified HSP70 in exosomes.

Mass spectrometry analysis of PTMs in extracellular vesicles released by insulinoma cells identified multiple poly-ubiquitinated proteins. Enrichment of exosomes in poly-ubiquitinated proteins was also demonstrated by an approach based on the use of FK1 antibody (which only binds poly-ubiquitinated proteins) and P4D1 (which labels poly- and mono-ubiquitinated proteins). Other studies suggest that exosomal proteins are preferentially mono-ubiquitinated or de-ubiquitinated, based on western analysis showing discrete ubiquitinated protein bands rather than smeared bands.

Ubiquitination has been shown to be important for the secretion of a novel type of extracellular vesicle, distinct from exosomes, called arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs). ARMMs directly bud from the plasma membrane upon interaction of the tumor susceptibility gene 101 protein (TSG101) with a PSAP motif in ARRDC1, which is localized through its arrestin domain at the plasma membrane. ARRDC1 in vesicles is ubiquitinated by the E3 ligase WW domain-containing protein 2 (WWP2). Down-regulation of WWP2 decreases ARRDC1 protein level in vesicles, and a PPXY-mutant of ARRDC1 strongly inhibits ARMM secretion, suggesting that ARRDC1 ubiquitination promotes ARRDC1 sorting into vesicles and ARMM secretion.

Another ubiquitin-like modifier called small ubiquitin-related modifier (SUMO) has been found to modify the exosomal-protein heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1). This modification affects the ability of this protein to export micro ribonucleic acids (miRNAs) into exosomes, probably by affecting its binding to miRNAs. hnRNPA1 in exosomes was also found to be modified, increasing its molecular weight by about 12 kDa on gel electrophoresis. This change in molecular weight of hnRNPA2B1 and other proteins has been shown before.

OTHER PTMs: PHOSPHORYLATION AND GLYCOSYLATION

Mass spectrometry analysis of extracellular vesicles also detects phosphorylated proteins. Phosphorylation and ubiquitination co-regulate sorting of Fas ligand (FasL) into secretory lysosomes by controlling its entry into MVBS. FasL contains a proline-rich domain (PRD) in the cytosolic tail to which tyrosine kinases, such as FGR, FYN, and LYN bind, and phosphorylation of tyrosine residues by these kinases enhances internalization to MVBS. The flanking regions of the PRD contain lysines, which are mono-ubiquitinated. Mutation of these lysines impairs the localization of FasL in MVBS, but mutation of the tyrosines does not affect mono-ubiquitination. Phosphorylation is thus not required for ubiquitination, but both PTMs are necessary for incorporation of FasL into to MVBS (Figure 1).

Phosphorylation is also involved in incorporation of the Ca2+-dependent phospholipid-binding protein Annexin A2 into exosomal membranes, through the action of raft-resident kinases, such as SRC or LYN on Tyr-23. Aberrant phosphorylation of the protein tau on threonine-181 promotes its incorporation into exosomes, resulting in the spreading of this abnormally processed protein in Alzheimer disease patients.

The protein γ-synuclein is transported in exosomes in its modified form. This modification consists of oxidation of Met-38 and Tyr-39, which confers prion-like properties and causes the formation of toxic aggregates. The spreading of these aggregates is in part mediated by the exosomal transport of oxidized-γ-synuclein to glial cells.

Carbohydrate modifications, involved in protein trafficking, cellular recognition, and communication of cells with their extracellular environment, have also been studied in extracellular vesicles. Vesicles of diverse cells types are enriched in proteins with high mannose, polyalactosamine, α,2,6-sialic acid, and complex N-linked glycans; in contrast, there is a comparative under-representation of specific glycan epitopes, such as terminal blood group A and B antigens. Exosome glycan profiles of different cell sources, such as T-cells, melanoma and colon cancer cells, and biological fluids like breast milk, are very similar, although they conserve some features of their parent membranes. The carbohydrate fingerprint detected in exosomes is less diverse than that observed in parent cells, but correspond to a conserved fraction of the parent cellular membrane that display a particular glycan profile.
cellular and exosomal carbohydrate signatures has been suggested to indicate different membrane microdomain origins of these vesicles (13). It has been described that polyLacNac and high mannose modifications associated with galectins and VIP36 are responsible for the oligomerization of glycoproteins that mediate their sorting into Golgi-derived vesicles (12, 52–55). Galectins and galectin-associated proteins have also been detected in exosomes so it is possible that glycosylation may also play a role in the sorting cargo into extracellular vesicles (12, 13).

Membrane anchors have also been shown to be important for the budding of vesicles derived from the plasma membrane. An N-terminal acylation tag serves as a signal for the import into extracellular vesicles (12, 13). It is possible that glycosylation may also play a role in the sorting cargo for the oligomerization of glycoproteins that mediate their sorting to indicate different membrane microdomain origins of these vesicles.

Post-translational modifications decorate proteins and drive their fate in cells by affecting multiple parameters including stability and localization. Different modifications can affect the same protein; sometimes competing with each other or being mutually exclusive, but in other cases can promote other modifications. Enzymes controlling PTMs additionally show very specific patterns of expression, activation, and subcellular localization, exponentially increasing the diversity and potentiality of cellular proteomes.

Different types of PTMs have been found in exosomal proteins; however, the role of these modifications in the localization of proteins into exosomes is not clear. The enigmatic role of ubiquitination, whose final consequences seem to differ depending on the target protein, is a particular case in point. The type of ubiquitination may also account for the fate of the modified protein, and could be a key determinant for its loading in exosomes. In some cases, ubiquitination seems to target the protein into MVBs destined for degradation, whereas sorting of proteins into MVBs that fuse with the plasma membrane to release exosomes seems to be ubiquitin-independent, clearly pointing to the existence of different types of MVB with different sorting mechanisms.

Specific protein modifications can reflect a particular pathological condition. The presence of modified proteins in exosomes can therefore make them invaluable tools for diagnosis, since modifications can be easily detected in exosomes obtained from body fluids without the need for invasive tissue biopsies.

ACKNOWLEDGMENTS
The authors thank Dr. S. Bartlett for assistance with English editing. This work was supported by SAF2011-25834 from the Spanish Ministry of Science and Innovation, INIDISEN-S2011/BMD-2332 from the Comunidades de Madrid, Red Cardiovascular RD 12-0042-0056 from Instituto Salud Carlos III (ISCIII), ERC-2011-AdG 294340-GENTRIS, and COST-Action BN1202. Olga Moreno-Gonzalo and Carolina Villarroya-Beltri were supported by FPU program (Spanish Ministry of Education).


33. Raiborg C, Stenmark H. The ESCRT machinery in endosomal sorting of ubiquitin... 10.37236/1.001641706


37. Bies J, Markus J, Wolff L. Covalent attachment of the SUMO-1 protein to... 1999.00948.x


Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 25 June 2014; paper published pending: 13 July 2014; accepted: 28 July 2014; published online: 11 August 2014.


This article was submitted to Immunotherapies and Vaccines, a section of the journal Frontiers in Immunology. Copyright © 2014 Moreno-Gonzalo, Villarroya-Beltri and Sánchez-Madrid. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original authors are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.