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Mechanisms of exosome biogenesis and secretion

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ABBREVIATIONS

AChE	Acetylcholinesterase
ALIX	ALG-2 interacting protein
BIG	Brefeldin A-inhibited guanine nucleotide exchange protein
BMDC	Bone marrow-derived dendritic cell
BSA	Bovine serum albumin
CHMP	Charged multivesicular body protein
DC	Dendritic cell
DGKA	Diacyl glycerol kinase α
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
EGFR	EGF receptor
EM	Electron microscopy
ESCRT	Endosomal sorting complex required for transport
FACS	Fluorescence- activated cell sorter
FasL	Fas ligand
GAP	GTPase-activating protein
GEF	Guanine nucleotide exchange factor
GM-CSF	Granulocyte-monocyte colony stimulating factor
GUV	Giant unilamellar vesicle
HSC70	Heat shock protein cognate 70
IFN-γ	Interferon-γ
IL-4	Interleukin 4
ILV	Intralumenal vesicle
LAMP	Lysosomal-associated membrane protein
LBPA	Lysobisphosphatidic acid
Mfge8	Milk fat globule - epidermal growth factor 8
MHC	Major histocompatibility complex
miRNA	microRNA
mRNA	messenger RNA
MVE	Multivesicular endosome
NK	Natural killer
NSCLC	Non small cell lung carcinoma
OVA	Ovalbumin
PBS	Phosphate-buffered saline
PE	Phopshatidyl ethanolamine
PFA	Paraformaldehyde
PLD	Phospholipase D
PLP	Proteolipid protein

siRNA	small interference RNA
shRNA	small hairpin RNA
SNARE	Soluble NSF attachment protein
STAM	Signal transducing adaptor molecule
STX	Syntaxin
TCR	T cell receptor
TCTP	Transcriptionally controlled tumor protein
TfR	Transferrin receptor
TNFR1	TNF receptor 1
tRNA	transfer RNA
TSG101	Tumor susceptibility gene 101
UBD	Ubiquitin binding domain
VAMP	Vesicular-associated membrane protein
Vps	Vacuolar protein sorting

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ABSTRACT

Exosomes are small membrane vesicles with sizes ranging from 30 to 100 nm in diameter, which are formed in multivesicular endosomes and secreted by most cell types. Numerous studies have focused on the biophysical and biochemical properties of exosomes, as well as the mechanisms of biogenesis and secretion of these vesicles. However, these aspects are not fully understood, which limits the analysis of the functions of exosomes in vivo. At least two mechanisms have been proposed for the biogenesis of exosomes: one would rely on the function of proteins involved in endosomal sorting, the ESCRT family (for "endosomal sorting complex required for transport"). Another mechanism would be independent of their activity. Once exosomes are formed in endosomes, their secretion requires the small GTPase RAB27A, as shown in a human cell line.

The objective of my PhD project was to gain insights into the molecular mechanisms that drive exosome biogenesis and secretion. A first study performed to analyze the function of Rab27a in murine cells allowed me to show the existence of different populations of exosomes, dependent or not on Rab27a for their secretion. A second study was aimed at analyzing the involvement of ESCRT proteins in exosome biogenesis in HeLa-CIITA cells. Seven molecules potentially involved in this process were identified on the basis of the screening of an RNA interference library directed against the different ESCRT proteins: the inactivation of HRS, STAM1 and TSG101 induced a decrease in exosome secretion, whereas the downregulation of CHMP4C, VPS4B, VTA1 and ALIX increased it. Gene expression of the different candidate proteins was inhibited and exosomes secreted by these cells were analyzed: we showed the heterogeneity of the secreted vesicles, as well as an alteration of their size and protein composition, as compared to control cells. In particular, the inactivation of ALIX induced an increase in the secretion of larger vesicles, and the selective enrichment of these vesicles in MHC class II molecules. Accordingly, I showed that both HeLa-CIITA and human primary dendritic cells inactivated for ALIX possess a higher expression of MHC class II molecules at the cell surface and in intracellular compartments. These results suggest that some members of the ESCRT family are involved in the exosome biogenesis and secretion pathway, and propose a potential role of ALIX in the trafficking of MHC class II molecules and in the modulation of the protein composition of exosomes.

RESUME

Les exosomes sont des vésicules membranaires de 30 à 100 nm de diamètre, formées dans les endosomes multivésiculaires et sécrétées par la plupart des cellules. Les propriétés biophysiques et biochimiques des exosomes ainsi que les mécanismes permettant leur biogénèse et sécrétion ont fait l'objet de nombreuses études. Cependant, ces derniers sont encore méconnus, limitant l'analyse des fonctions des exosomes *in vivo*. Au moins deux mécanismes ont été proposés pour la biogénèse des exosomes : un mécanisme nécessiterait l'action de protéines impliquées dans le tri endosomal, les ESCRT (« endosomal sorting complex required for transport »). Un autre mécanisme serait indépendant de leur fonction. La sécrétion des exosomes, une fois générés dans les endosomes, requiert la petite GTPase, Rab27a, comme montré dans un modèle cellulaire humain.

Mes travaux de thèse ont porté sur l'étude des mécanismes moléculaires impliqués dans la biogénèse et la sécrétion des exosomes. Une première étude visant à analyser la fonction de Rab27a dans des cellules murines, m'a permis de mettre en évidence l'existence de différentes populations d'exosomes, dont la sécrétion dépend ou non de Rab27a. Une deuxième étude a eu pour objectif d'analyser l'implication des ESCRT dans la biogénèse des exosomes dans des cellules HeLa CIITA. Le criblage d'une librairie d'ARN d'interférence dirigés contre les différentes protéines ESCRT, a permis l'identification de 7 molécules potentiellement impliquées dans cette voie : HRS, STAM1, TSG101, leur inactivation induisant la diminution de la sécrétion des exosomes. L'inactivation de CHMP4C, VPS4B, VTA1 et ALIX, au contraire, l'augmente. L'inhibition de l'expression de ces candidats suivie de l'analyse des exosomes sécrétés a démontré l'hétérogénéité des vésicules sécrétées, et une modification de leur taille et de leur composition protéique par rapport aux cellules contrôle. Plus particulièrement, l'inactivation d'ALIX induit une augmentation de la sécrétion d'exosomes de plus grande taille, et l'enrichissement sélectif en molécules de CMH de classe II. En accord, j'ai montré que les cellules inactivées pour ALIX, aussi bien des cellules HeLa que des cellules dendritiques humaines ont une plus forte expression de CMH de classe II à la surface et dans des compartiments intracellulaires. Ces résultats suggèrent l'implication de certains membres de la famille ESCRT dans la voie de biogénèse et sécrétion des exosomes, ainsi qu'un rôle potentiel d'Alix dans le trafic des molécules CMH de classe II, et dans la modulation de la composition protéique des exosomes.

INTRODUCTION

INTRODUCTION

Multicellular organisms are complex entities that have developed various cellular lineages to achieve the diversity of biological functions. Cells undergo division, differentiation, apoptosis, and perform specific tasks (such as antigen presentation by immune cells, pigment production by melanocytes, etc.) in a highly regulated fashion. Intercellular communication allows the coordination of such functions, and this constitutes the basis of development, tissue homeostasis and repair, and immunity among others. It involves molecular mediators, some of which must be recognized by specific receptors in the recipient cell in order to induce a modification of its physiological state. Cells can exchange information via direct cell-to-cell contact, by means of gap junctions (intercellular channels that allow the passage of ions and small metabolites), neuronal synapses or immune synapses (highly organized structures that assemble at the zone of contact between immune cells). Alternatively, communication can occur via molecular messengers that are released in the extracellular milieu and must travel to encounter the recipient cell. Soluble molecules have been extensively studied in this context, including hormones, growth factors, neurotransmitters, cytokines and chemokines.

More recently, several research groups have uncovered another type of messengers between cells. Different cell types release vesicles to the extracellular space: they consist of small volumes of cytosol enclosed by a lipid bilayer. The types of vesicles that may be released depend on the cell type and its physiological state (differentiation, activation, transformation, stress). At first, membrane vesicles were considered as cell debris or as a consequence of cell death (Chatterjee and Kim, 1977; De Broe et al., 1977). It is only recently that these elements, including ectosomes, microvesicles, prostasomes, and exosomes have started to be regarded as mediators of intercellular communications in a variety of tissues, as further research has revealed a series of important functions that these nanometer-sized structures might carry out. In particular many different functions have been attributed to exosomal vesicles and even therapeutical strategies start to be developed exploiting exosomes.

In the past thirty years our knowledge of exosomes has considerably grown, although many aspects of this new and exciting field of research still remain elusive. Particularly, the physiological relevance of exosomes *in vivo* is nowadays unknown, since the answer to this question requires a detailed knowledge of the mechanisms and molecular effectors involved in the exosome secretion pathway. We have thus focused our work on the molecular mechanisms that lead to the biogenesis and the secretion of exosomes. This will provide tools to better control exosome secretion by cells and thereby allow scientists to unravel the function(s) of exosomes released under physiological conditions.

I will start by describing the different types of extracellular vesicles that can be secreted by cells. I will then focus on the current knowledge of exosomes, from their discovery to their biophysical and biochemical characteristics, including an overview of the isolation and detection methods we dispose of today. I will then present the different intracellular effectors that have been linked to the biogenesis and the secretion of exosomes.

FIGURE 1 – Different types of vesicles released by cells.
Exosomes are formed in multivesicular endosomes by inward budding of the limiting membrane, and are secreted by fusion of these compartments with the plasma membrane: this causes exosomes to retain a similar membrane orientation as the plasma membrane. This is due to two inward budding events: one leading to the formation of early endosomes by endocytosis of the plasma membrane (note that the extracellular portion of the membrane, in red, is on the inside of the compartment), and the other one leading to the budding of exosomes towards the lumen of the multivesicular endosomes (as seen in the figure this causes the inversion of the membrane, the extracellular portion now being on the outside of the vesicles).

Other membrane vesicles, such as microvesicles, exosome-like vesicles and membrane particles, are secreted by direct budding from the plasma membrane. Enveloped viruses can bud either at the plasma membrane, or towards the lumen of multivesicular endosomes and then secreted by exocytosis like exosomes.

From Théry et al., 2009, Nature Reviews Immunology

I- Exosomes: history and current definition

1.1- Different types of secreted membrane vesicles

Cells can secrete different types of membrane-bound vesicles, and these have been given many different names throughout the literature: membrane vesicles, ectosomes, exovesicles, membrane particles, exosomes, argosomes, microvesicles, nanoparticles, exosome-like vesicles, tolerosomes, prostasomes, etc. We may categorize secreted vesicles in terms of their intracellular origin (Gyorgy et al., 2011; Thery et al., 2009). Vesicles such as exosomes are formed in endosomal compartments and released upon fusion of these compartments with the plasma membrane. Other vesicles, however, may be released directly from the plasma membrane by budding and shedding: these include microvesicles, ectosomes, and membrane particles. As to exosome-like vesicles, their exact cellular origin is still unknown. Dying cells can release vesicles referred to as apoptotic vesicles, which are distinct from exosomes (Thery et al., 2001); it is important to note that culture conditions harmful to cells can cause the accumulation of these particles in the conditioned medium. A brief description of vesicles released by plasma membrane budding follows, and is summarized in **Figure 1** and **Table 1**.

Microvesicles are released by shedding from the plasma membrane (Heijnen et al., 1999); ultracentrifugation at 10,000 x g is commonly employed to pellet these particles, although several groups have used different g forces to do so, possibly in an effort to pellet also the smaller microvesicles: 25,000 x g for plasma microvesicles (Grant et al., 2011), 28,000 x g for platelet-derived microparticles (Baj-Krzyworzeka et al., 2002) and 50,000 x g to pellet microvesicles from tumor cells (Baj-Krzyworzeka et al., 2007). Observation by electron microscopy (EM) shows that their size ranges from 100 nm to 1 μm, and they have an electron-dense core surrounded by membrane (Muralidharan-Chari et al., 2009). They can be detected by flow cytometry after platelet activation, and expose phosphatidylserine at the outer leaflet of the vesicle surface (as evidenced by the binding of Annexin V). The protein content of microvesicles differs from that of exosomes: less enriched in CD63 than exosomes, they carry selectins and integrins (the latter can also be found in exosomes).

Neutrophils secrete ectosomes (Gasser et al., 2003; Hess et al., 1999) by a process termed "ectocytosis"; these vesicles are enriched in cholesterol, diacylglycerol and phosphatidyl-

TABLE 1 - Physicochemical characteristics of different types of secreted vesicles.
Cells can secrete different types of membrane-bound vesicles, which have specific biophysical properties such as size and density, and biochemical properties such as protein and lipid content. They sediment at a different centrifugal speed, and have a particular appearance when observed by electron microscopy. Secreted vesicles may be classified according to their intracellular origin: internal compartments that fuse with the plasma membrane (specific of exosomes), or shedding from the plasma membrane (microvesicles, ectosomes, membrane particles).
From Théry et al., 2009, Nature Reviews Immunology

FIGURE 2 – Exosomes and microvesicles by electron microscopy

A, B) Melanoma cells were fixed and embedded in epon for conventional electron microscopy. A) Multivesicular endosome. B) Secretion of exosomes. Note that the secreted vesicles have the same morphology and size of the intraluminal vesicles of the multivesicular endosomes. C) Breast cancer cells fixed and embedded in epon for conventional electron microscopy. Large membrane vesicles bud from the plasma membrane. D, E) Isolated exosomes and microvesicles were contrasted and embedded in a mixture of uranyl-acetate and methylcellulose. D) Exosomes were immunogold labeled with antibodies anti-CD63 and Protein A gold 10 nm.

Magnification bars 200 nm, except in C: 1000 nm.

From Lo Cicero and Raposo, 2013, Emerging Concepts of Tumor Exosome–Mediated Cell-Cell Communication (Chapter 1: The Cell Biology of Exosomes: Historical and Perspectives)

serine. They express complement receptor 1 (CR1) and proteases (myeloperoxidase and elastase) but no CD63 was detected; by EM they appear as bilamellar structures with sizes from 70 to 300 nm diameter. They are pelleted at 160,000-200,000 x g.

Membrane particles (Marzesco et al., 2005) are 50-80 nm membrane vesicles, enriched in CD133 (prominin-1, a cell surface glycoprotein) but not in CD63, which suggests they are originated in the plasma membrane rather than in MVEs. They are pelleted at 110,000-200,000 x g.

Particles referred to as exosome-like vesicles (Hawari et al., 2004) are small (20-50 nm diameter) and have an irregular shape as visualized by EM. They sediment by ultracentrifugation at 175,000 x g, and carry the TNF receptor type I (TNFR1). Even though their lipid composition is unknown, it has been established that they do not contain lipid rafts.

Currently, there is no consensus in the field as to a proper nomenclature and the exact definition of each type of vesicle, mainly because different research groups employ different means to purify and characterize them. One of the challenges that the newly created International Society for Extracellular Vesicles (www.isev.org) faces is to establish guidelines for the purification of each type of vesicle, and also a definition and a minimal set of requirements (protein and lipid composition, size, density, etc.) to verify which type of vesicle is enriched in a particular preparation, and to distinguish vesicles secreted by plasma membrane shedding from those derived from endocytic compartments, such as exosomes. This will be an important step towards a more accurate description of the function that each type of vesicle may have in physiological conditions.

1.2- Exosomes: historical considerations

1.2.1 Discovery of exosomes

Exosomes were first described in the mid-eighties by the groups of P. Stahl and R. Johnstone (Harding et al., 1983); (Pan et al., 1985), while studying the maturation of red blood cells, namely of reticulocytes into erythrocytes, which can be monitored by the loss of the transferrin receptor (TfR), originally found in the plasma membrane, into the extracellular

FIGURE 3 – Discovery of exosomes secreted by reticulocytes.

Small membrane vesicles were detected by immuno-electron microscopy in the lumen of multivesicular endosomes (top micrograph), as well as in the process of being released from the cells by exocytosis (bottom micrograph). The gold particles (20nm) indicate the presence of the transferrin receptor; note the presence of the receptor only on the 50-nm vesicles and neither on the multivesicular endosome limiting membrane or the plasma membrane.

From Pan et al., 1985, The Journal of Cell Biology

medium. The way in which the TfR is released was unknown, although the location of the receptor in intracellular compartments after internalization had already been described. Using transferrin bound to gold particles (Harding et al., 1983), or anti-TfR antibodies (Pan et al., 1985), both groups implemented an EM approach to follow the fate of the endocytosed receptor during its trafficking in the cell and subsequent release. They observed the location of TfR in early endosomes, and at later time points in multivesicular endosomes (MVEs), and found that most of the gold staining corresponding to the receptor was located not on the surface of the intracellular compartments, but on smaller internal "bodies" around 50-nm diameter, which were released upon fusion of the MVEs with the plasma membrane of the cells (**Figure 3**).

These studies suggested that this novel form of secretion was the way that plasma membrane components (such as the TfR) were discarded from maturating reticulocytes. This was confirmed a few years later, when another article (Johnstone et al., 1987) showed that the secreted vesicles, analyzed after recovery by ultracentrifugation at 100,000 x *g*, had a series of enzymatic or binding activities that were normally present in the plasma membrane of reticulocytes, thereby implying that membrane proteins other than the TfR were also discharged in this manner. In this article, the term "exosome" was used for the first time to describe small membrane vesicles formed by vesiculation of intracellular endosomes and released by exocytosis from the reticulocytes; the word had previously been used (Trams et al., 1981) to designate secreted vesicles of heterogeneous sizes (including one population of large vesicles with diameters between 500 and 1000 nm, and a second, smaller population around 40-nm diameter), but the manner in which the normal and neoplastic cells used in this study released these vesicles was unknown.

1.2.2 Milestones in exosome biology

In the following years, exosomes were marginally studied, mostly in reports related to the differentiation of red blood cells, and the main conclusion at the time was that exosomes were a means for cells to dispose of proteins that were no longer necessary. The publication of a groundbreaking paper in 1996 sparked a renewed interest in the field of exosome biology, since it suggested for the first time that exosomes might be important mediators of intercellular communication, instead of mere transporters of obsolete molecules. G. Raposo

and colleagues (Raposo et al., 1996) showed that Epstein-Barr virus (EBV) transformed B cell lines were able to secrete exosomes that were enriched in MHC class II molecules. The authors demonstrated the endosomal origin of the secreted vesicles by showing fusion profiles of multivesicular MIIC compartments -late endocytic compartments enriched in MHC class II molecules, where the loading of antigenic peptides occurs- with the plasma membrane, and subsequent release of the internal vesicles of these compartments. The concomitant secretion of a previously internalized tracer (BSA gold) further reinforced the endocytic origin of the release vesicles. Furthermore, they show by surface biotinylation that the protein composition of secreted exosomes was different from that of the plasma membrane, thus ruling out the possibility that the vesicles were produced by shedding of the plasma membrane. Importantly, in both human and murine models, exosomes released by B lymphocytes have the capacity to stimulate specific CD4+ T cell clones *in vitro*, suggesting a possible role of exosomes as transporters of MHC class II-peptide complexes between cells of the immune system. Zitvogel et al. took these findings one step further two years later (Zitvogel et al., 1998), by demonstrating the release of exosomes by human dendritic cells (DCs), and the ability of tumor peptide-pulsed DC-derived exosomes to suppress the growth of established tumors *in vivo*. These potential roles as mediators of immune responses, and the suggestion of a possible use of exosomes as immunotherapeutic agents has lead to a myriad of articles related to the immune function of exosomes in vitro and in vivo (reviewed in (Thery et al., 2009) and (Bobrie et al., 2011), as well as several clinical trials involving the use of exosomes as cell-free vaccines (reviewed in (Chaput and Thery, 2011).

Since then, many different cell types have been discovered to secrete exosomes *in vitro*: epithelial cells (van Niel et al., 2001), neurons (Faure et al., 2006), Schwann cells (Fevrier et al., 2004), and tumor cells (Wolfers et al., 2001) among others. Furthermore, exosomes have been purified from numerous body fluids, such as urine (Pisitkun et al., 2004), saliva (Ogawa et al., 2008), bronchoalveolar fluid (Admyre et al., 2003) seminal fluid (Gatti et al., 2005), amniotic fluid (Keller et al., 2007), breast milk (Admyre et al., 2007), tumor effusions (Andre et al., 2002), cerebrospinal fluid (Street et al., 2012) and blood serum (Taylor et al., 2006) and plasma (Caby et al., 2005), thus proving that exosomes are also secreted *in vivo*.

The year 2007 marked a breakthrough in the field: the group of J. Lötvall discovered that exosomes, in addition to lipids and proteins, carry nucleic acids (Valadi et al., 2007).

Exosomes secreted by mouse and human mast cell lines were purified by centrifugation, and the analysis of nucleic acid content revealed that exosomes contain no DNA, but do contain functional messenger RNA (mRNA) transcripts enclosed inside the vesicles, as well as microRNA (miRNA). Strikingly, when mouse exosomes were transferred to human cells, mouse proteins were detected in these cells, which suggests that mRNA shuttled via exosomes to different cells can be translated to proteins. This important work has provided scientists from the field with a new perspective on the possible roles exosomes might play, and has proposed exosomes as potential vectors for gene therapy.

1.3- Definition of exosomes

1.3.1 Endosomal origin

Exosomes are a distinct type of secreted vesicle, and differ from those described previously mainly by their intracellular origin (Harding et al., 1983; Pan et al., 1985): they are formed by budding of the limiting membrane of "sorting" vacuolar endosomes towards the lumen of these compartments, thus forming intralumenal vesicles (ILVs); the endosomes are then referred to as MVEs. Upon fusion of the MVEs with the plasma membrane, the internal vesicles are released to the extracellular medium and are then named exosomes. As shown in **Figure 1**, the biogenesis of exosomes entails a specific topology of the vesicles: exosomes display an identical membrane orientation as that of the plasma membrane (which can be assessed in the figure by following the red line: note how it is directed towards the extracellular space in both cases), and enclose a fraction of the cell cytoplasm (represented with beige color in the figure), including soluble proteins.

1.3.2 Physical properties

Due to their endosomal origin, exosomes possess hallmarks of ILVs of the MVEs, such as their size, which ranges from 30 to 100 nm in diameter, and reflects the heterogeneity of purified exosomes. Exosomes float at a density between 1.13 and 1.19 g/mL (Thery et al., 2006), and can be pelleted by ultracentrifugation at 100,000 x g or higher. Importantly, the

comparison of these properties with those of other vesicles (described above, see **Table 1**) illustrates the difficulty of separating vesicles from different origins, since sizes and sedimentation speeds of the different types of vesicles often overlap.

1.3.3 Purification protocols

A general protocol used in initial articles to purify exosomes (Johnstone et al., 1987; Raposo et al., 1996) and described in detail in (Thery et al., 2006), is based on centrifugation. It involves several consecutive steps: a first centrifugation at 300 x g aims at separating floating cells. The supernatant is then centrifuged at 2000 x g to eliminate dead cells and large cell debris. A subsequent step of ultracentrifugation at 10,000 x g is used to pellet cell debris, as well as other types of secreted vesicles, such as microvesicles, which are larger in size. The final ultracentrifugation at 100,000 x g allows the recovery of exosomes. As mentioned before, it is important to note that this step only allows enrichment in exosomes and not a proper purification, since other vesicles of similar size as well as protein aggregates are likely to sediment at the same speed.

The protocols used to purify exosomes -or at least to enrich a given preparation in this type of vesicles- can vary considerably from one laboratory to another. Centrifugation is probably the most commonly used technique, but many different strategies have been used so far, some of which will be discussed in the following paragraphs.

An improved way to separate exosomes from other contaminants can be to perform continuous sucrose density gradients (Escola et al., 1998; Raposo et al., 1996), usually after sedimentation at 100,000 x g. Exosomes float at a density that generally ranges from 1.13 and 1.19 g/mL, and these densities slightly vary between different secreting cell types. This technique ensures the elimination of different contaminants such as protein aggregates and soluble proteins. The exosomes will thus be recovered in different fractions of the gradient, and the fractions can be pooled for further analysis, or analyzed independently.

Another group (Taylor et al., 2006) has used size exclusion chromatography followed by a sucrose density gradient centrifugation, and exosomes were obtained by a final ultracentrifugation at 100,000 x g. This also allows the elimination of contaminating proteins, but the entire procedure may not succeed in separating large vesicles from smaller ones. Some groups have employed a filtration step (through 0,22 micron filters) to eliminate

cells, debris and larger vesicles (Ostrowski et al., 2010; Thery et al., 2001; Valadi et al., 2007), prior to ultracentrifugation to obtain pelleted vesicles. This method can however induce the loss of aggregates of small vesicles.

Protocols based on the use of antibody-coated magnetic beads (Clayton et al., 2001) or latex beads (Ostrowski et al., 2010) were also developed. They rely on the enrichment of exosomes in certain surface proteins, which are used to trap exosomes on beads and analyze them by flow cytometry. This technique will allow a specific recovery of exosomes if the proper markers are used (and these may vary between different cell types), but prevents their use in further experiments due to the difficulty of extracting them from the beads; it can thus only be used to identify a particular molecular signature.

An alternative method to produce clinical-grade exosomes (Lamparski et al., 2002) involves ultrafiltration of the cell-conditioned medium followed by centrifugation in sucrose deuterium oxide (D_2O) cushions. This requires particular laboratory equipment (filters, pumps, etc.), and is suitable to isolate exosomes from large volumes of cell supernatant, or to obtain exosomes under strict regulatory guidelines.

Recently, commercially available methods that promote fast and simple exosome purification procedures (such as Exoquick, http://www.systembio.com/microrna-research/exoquick-exosomes/technical-details) have been released. Before they become of standard use in laboratories, they should go through a thorough characterization process to ensure that the obtained vesicles are indeed exosomes, and that they are not contaminated by the presence of other vesicles or even large membrane debris.

It is important to consider that regardless of the protocol that is used, each technique must be validated for any given cell type, or biological fluid as a source of exosomes, to confirm the identity of the purified vesicles. This requires the use of a combination of several methods, which are described below.

1.3.4 Detection methods

Due to their size in the nanometer range, transmission electron microscopy is the only technique that allows direct observation of the size and morphology of exosomes. Pelleted vesicles, resuspended in a small volume of buffer, are deposited on grids, fixed, embedded and contrasted using a mixture of methyl cellulose and a heavy metal, uranyl acetate,

before observation: exosomes display a characteristic cup-shape appearance. It is important to note that this morphological feature is certainly an artifact due to the fixation/contrast step, as exosomes observed by cryo-EM (a technique in which samples are vitrified in liquid ethane to prevent the formation of ice crystals that can alter the ultrastructure of cells and membranes) have a round shape (Conde-Vancells et al., 2008). Evaluation of the presence of selected proteins at the surface of exosomes can be achieved through the use of immuno-EM: specific antibodies and gold particles of different sizes allow the identification of a few proteins in the same sample simultaneously. The purity of exosome preparations can also be assessed with this method, since the presence of debris and other membrane vesicles of larger size or more electron-dense can be detected.

Classical biochemistry methods, such as gel electrophoresis followed by Coomassie blue staining, and western blotting, provide more information as to the protein content of the obtained vesicles. The former technique (Thery et al., 2006) is used to compare the pattern of bands between pelleted vesicles and whole cell lysates: a different pattern is indicative of a good purity of the exosomal pellet, since it rules out the presence of cell debris. Western blotting is routinely used to evaluate the presence of particular proteins in the 100,000 x *g* pellet and compare it to cell lysates (Raposo et al., 1996; Thery et al., 1999; Zitvogel et al., 1998): exosomes are enriched in some proteins such as MHC class II and TfR (depending on the cell type), but do not have others like invariant chain or calnexin (an endoplasmic reticulum-specific marker).

Another technique that allows quantitative analysis of protein content is flow cytometry. However, the limit of detection of a typical flow cytometer is around 200 nm, and as mentioned earlier exosomes are smaller and would thus appear as background noise. The adsorption of exosomes directly on beads (Thery et al., 2006; Thery et al., 2001) has been used to detect different proteins with the use of fluorescently labeled specific antibodies. This can be applied when the exosome preparation is highly pure. An improvement has been to use beads coated with antibodies directed against proteins that are known to be enriched in exosomes (as described above in the purification methods section, (Clayton et al., 2001; Ostrowski et al., 2010), which will increase the chances of analyzing exclusively exosomes.

An alternative protocol involving the use of high-resolution flow cytometry was recently developed by the group of M. Wauben (Hoen et al., 2012). This method combines the use of

a BD Influx flow cytometer (that can be optimized to detect particles as small as 100 nm in diameter), and fluorescently-labeled vesicles. Interestingly, heterogeneous samples can be characterized with greater detail with this method.

Atomic force microscopy is another technique that has been recently used to detect membrane vesicles (Sharma et al., 2010). Exosomes can be studied at the single-particle level to acquire structural information, as well as to detect a specific protein marker (such as CD63).

A new instrument called NanoSight has been designed to measure the size distribution and concentration of nanoparticles (Dragovic et al., 2011) and www.nanosight.com). It is based on the principle that particles in suspension move under Brownian motion, and when they scatter light their movement can be tracked individually by a software (Nanoparticle Tracking Analysis or NTA) that can then calculate their diameter by statistical methods. Fluorescent particles may also be detected with the use of two lasers with different wavelengths. This method has the advantage of being a fast and simple way of analyzing large numbers of particles simultaneously. However, appropriate parameters during the acquisition of the sample (such as sample dilution, camera shutter speed and gain, and length and number of videos), as well as during the analysis (the detection threshold is set for each sample by the operator) must be determined so as to obtain reproducible results. The results are less accurate when samples are polydispersed, i.e. when particles of many different sizes coexist in the sample, which is the case of secreted vesicles. In addition, this technique does not allow the morphological identification of different types of secreted vesicles (in other words, it cannot distinguish between an exosome and a microvesicle of similar sizes).

These various methods provide different information about the purified vesicles, in terms of size, morphology and biochemical composition. It is thus crucial to use these techniques in a complementary fashion to adequately define these vesicles.

FIGURE 4 – Protein composition of exosomes.
The proteins most commonly found on exosomes released by different cell types are depicted here. This representation summarizes data obtained from 15 proteomic studies performed on exosomes purified from cultured cells and body fluids.
From Théry et al., 2009, Nature Reviews Immunology

1.3.5 Molecular composition of exosomes

a) Proteins

Since their initial description, many efforts have been made to better characterize the protein composition of exosomes. EM analysis first revealed the presence of TfR associated to exosomes released by reticulocytes (Harding et al., 1983; Pan et al., 1985). Afterwards, western blotting in combination with EM was used to show the enrichment of exosomes from B lymphocytes (Raposo et al., 1996) in MHC class II but not in TfR, whereas exosomes from dendritic cells (Zitvogel et al., 1998) carry MHC class I and II molecules, as well as TfR and tetraspanins (CD63 and CD82). The next step in the determination of the protein composition of exosomes involved peptide mass mapping to identify exosomal proteins such as MFG-E8 (lactadherin), CD9, and HSC73. Since then many proteomic approaches have been used by different groups, to better characterize exosomes secreted by different cell types in culture: DCs (Thery et al., 2001), B lymphocytes (Wubbolts et al., 2003), mast cell lines (Skokos et al., 2001), intestinal epithelial cells (Van Niel et al., 2003), hepatocytes (Conde-Vancells et al., 2008), cortical neurons (Faure et al., 2006), adipocytes (Aoki et al., 2007), Mov cells (immortalized Schwann cells from mice overexpressing the prion protein, Fevrier et al., 2004), melanoma cells (Mears et al., 2004), colorectal cancer cells (Choi et al., 2007); as well as exosomes purified from various body fluids: seminal fluid (Gatti et al., 2005), breast milk (Admyre et al., 2007), urine (Pisitkun et al., 2004).

These studies show that exosomes contain a specific set of proteins, some of which are dependent on the cell type that secretes them, while others are found in most exosomes regardless of the cell type (**Figure 4**). These include proteins involved in membrane trafficking (Rab GTPases such as Rab5, and annexins) and MVE biogenesis (such as clathrin, Alix and TSG101), adhesion molecules (Mfge8, integrins, and tetraspanins such as CD63, CD81, CD9 and CD82), chaperones (HSC70, HSP90), cytoskeleton molecules (actin, myosin, tubulin), and proteins associated to lipid rafts (flotillin-1) among others. Additionally, cell type-specific proteins have been evidenced in exosomes released by B cells (B cell receptor) and DCs (MHC class II, CD86, ICAM-1), among others. It is important to note that proteomic studies have shown the presence of proteins from endosomes, the plasma membrane, and the cytosol in vesicles secreted by all cell types, but proteins from the nucleus,

mitochondria, endoplasmic reticulum and the Golgi apparatus are mostly absent. This highlights the specificity of formation of these vesicles, and shows they represent a specific subcellular compartment, and not a random array of cell fragments.

Proteomic studies do not allow quantification of the identified proteins; it has thus not been possible so far to compare the relative levels of each protein within exosomes, and determine which are major and minor components. Nevertheless, some of these proteins are specifically enriched in exosomes as compared to cell lysates (as determined mainly by western blotting); these molecules have thus been considered as markers of exosomes (CD63, CD9, CD81, Alix, TSG101, HSC70, Mfge8, MHC class II), and have been used to confirm the identity of purified vesicles. Importantly, few articles have compared side by side different types of vesicles (Heijnen et al., 1999); (Thery et al., 2001), and it is therefore unknown if markers considered as specific of exosomes are not found in other vesicles as well.

b) Lipids

Fewer studies have been performed to analyze the lipid composition of exosomes, and show that it differs from that of the plasma membrane. Exosomes from B cells (Wubbolts et al., 2003) contain phosphatidyl ethanolamine, phosphatidyl choline, phosphatidyl inositol, phosphatidyl serine, and are enriched (compared to total cell membranes) in cholesterol, sphingomyelin, and ganglioside GM3, lipids commonly found in detergent-resistant membranes such as lipid rafts. By contrast, exosomes do not contain cardiolipin (a lipid mostly found in mitochondria) or lysobisphosphatidic acid (LBPA). Another study performed with exosomes from mast cells and DCs (Laulagnier et al., 2004b) found an enrichment in the same type of lipids, with the exception of cholesterol in vesicles derived from mast cells, suggesting that they do not possess lipid rafts, and that the lipid composition may vary between exosomes from different cells. It was also shown that the exosomal membrane does not have an asymmetrical distribution of the lipid phosphatidyl ethanolamine (PE), whereas PE is asymmetrically distributed in the inner leaflet of the plasma membrane; this might be due to the lack of flippase (a transmembrane lipid transporter which actively maintains phospholipid asymmetry in the plasma membrane) in exosomes and possibly MVEs. Trajkovic and colleagues (Trajkovic et al., 2008) uncovered a clear enrichment in

ceramide of exosomes released by oligodendrocytes, which they linked to the biogenesis of these vesicles. Finally, the presence of lipid rafts on exosomes from different origins (reticulocytes, the lymphoid B-cell line Daudi and the erythroleukemia cell line K562) was determined by the presence of typical lipid raft components associated to detergent-resistant membranes; these components include the ganglioside GM1, the Src tyrosine kinase Lyn, GPI-anchored protein acetylcholinesterase (AChE), stomatin and flotillin-1 (de Gassart et al., 2003).

c) Nucleic acids

As described before, nucleic acids were found in exosomes secreted by mast cells in vitro (Valadi et al., 2007): they contained, confined within the vesicles, around 1,300 different mRNA transcripts, with different levels as compared to the donor cells and which were functional for the translation of proteins. The authors also proved that the mRNA could be translated when exosomes were transferred to other cells, as evidenced by the presence of mouse proteins in human cells incubated with mouse exosomes; suggesting a function of exosomes as shuttles of genetic information between cells. These exosomes also contained miRNA, short 18-22 nucleotide sequences that mediate gene silencing, which were found at different levels as those present in secreting cells.

Further studies have shown that mRNA and miRNA can be found in exosomes present in biological fluids (Michael et al., 2010; Rabinowits et al., 2009), and suggest that the expression patterns of these molecules could be useful as biomarkers for the diagnosis and prognosis of several pathologies. Other reports have revealed potential functions of secreted vesicles carrying nucleic acids in tumor growth and cell migration (Skog et al., 2008; Zhang et al., 2010), however the vesicle preparations contained both exosomes and other types of secreted vesicles such as microvesicles. This may also be the case for many other publications, in which the purification protocols or the characterization of the purified vesicles cannot confirm their identity, thus hindering our understanding of exosomal-associated nucleic acids and their potential uses and functions.

More recently, progress was made in identifying the different species of RNA that are present in secreted vesicles: in addition to miRNA and mRNA, which are detected by simpler and more easily available techniques such as array hybridization, other small RNA have been

identified by Next-Generation Sequencing techniques in DC-derived exosomes (Nolte-'t Hoen et al., 2012). Several small non-coding RNA were thus found, including vault-RNA, Y-RNA and selected tRNA among the most abundant species; and many of these exosomal RNA were enriched relative to cellular RNA, indicating a specific release of certain species via exosomes. Consequently, gene regulatory functions of RNA contained in exosomes may be extended to species other than mRNA and miRNA.

Anther study has investigated the targeting of miRNA species in a heterogeneous population of released vesicles (Palma et al., 2012). Breast cancer cells release a variety of vesicles, with sizes ranging from 20 to over 400 nm in diameter. These heterogeneous particles were subjected to a sucrose density gradient centrifugation, and the vesicles obtained in each fraction (i.e. of different densities) were shown to be enriched different species of miRNAs and other small RNAs. An original approach termed "differential buoyant velocity centrifugation" that separates vesicles according to density and size was used, and indicated that miRNAs associate to specific subtypes of vesicles, which might be of consequence for the use of these as diagnostic or prognostic tools for different pathologies.

The results from various studies on the molecular composition of exosomes were assembled in a database named "Exocarta" (Mathivanan and Simpson, 2009; www.exocarta.org), to facilitate the access to information about the molecular composition of exosomes, including data on proteins, nucleic acids, and lipids. More recently, these data were combined with those available on other secreted vesicles into a more comprehensive database named "Vesiclepedia" (www.exocarta.org/vesiclepedia); this compendium is still in progress and will contribute to the harmonization of the nomenclature of the different extracellular vesicles and their purification methods.

1.4- Proposed functions of exosomes

1.4.1 Interaction of exosomes with recipient cells

The earliest records of exosomes described them as means to release unwanted material from the cells. Recent advances have contested this notion, suggesting the concept of

exosomes as messengers involved in intercellular communication. Exosomes secreted by one cell must therefore interact with a recipient cell to induce changes in its physiology. The discovery of how exosomes interact with target cells has been held back by the difficulty to visualize exosomes, since they are under the limits of detection of microscopes (around 200 nm) due to their small size. Thus, the details of this interaction are still unknown. However, this process must involve a first step of binding of exosomes to the cell surface, which should involve specific receptors on both the exosome and the plasma membrane. Integrins and other adhesion molecules on the surface of exosomes (and on the plasma membrane) are good candidates to perform this step. A study regarding the potential role of exosomes secreted by intestinal epithelial cells on the immune system showed that exosomes secreted by these cells interacted preferentially with immature DCs and not with B or T lymphocytes (Mallegol et al., 2007), thus reinforcing the idea that specific receptors are involved in the interaction of exosomes with a particular type of recipient cell.

In some cases this first step might be sufficient to induce changes in the physiological state of the recipient cells, for instance during the presentation of MHC-peptide complexes on the surface of antigen-presenting cell-derived exosomes, to antigen-specific T cells. In other cases exosomes must be internalized to exert their function (one example would be gene silencing or expression induced in the recipient cell by nucleic acids contained inside the exosomes), which can be achieved by receptor-mediated endocytosis of individual vesicles, or alternatively by phagocytosis of exosome aggregates.

Another possibility of entry of exosomes to the receiving cell is the fusion of the exosome with either the plasma membrane or the limiting membrane of endocytic compartments (if exosomes are internalized in cells through endocytosis), which has been explored in two recent articles. One approach involved a fusion assay based on the use of the self-quenched fluorescent R18 lipid probe, which upon dilution increases its fluorescence (Parolini et al., 2009). The authors first proved that exosomes were internalized by cells, and appeared located in early endosomes and lysosomes, but not the Golgi apparatus. Then, to test the hypothesis that exosomes were internalized by fusion, they added R18-labeled exosomes to cell cultures and observed an increase in fluorescence, thus evidencing the lipid mixing (and ensuing dilution of R-18) that was occurring between the two membranes. By confocal microscopy, the authors visualized co-localization of exosomes and both the plasma and intracellular membranes, which therefore does not allow to conclude if the fusion event

occurs between exosomes and the plasma membrane, or between exosomes that have been endocytosed and the endocytic membrane. Different pH conditions were subsequently tested as part of the fusion assay, and showed that fusion was enhanced under an acidic pH, which is a feature of the tumor microenvironment and is thus representative of what occurs in the tumor mass.

Another article analyzed the capacity of murine DCs to internalize exosomes, and also explored the possibility of membrane fusion as a mechanism for exosome entry in these cells (Montecalvo et al., 2012). Labeling bone marrow-derived DCs (BMDC)-derived exosomes with pHrodo, a dye that fluoresces red at the phagosomal pH, and incubation with acceptor BMDCs was used to test the ability of DCs to endocytose exosomes: acceptor DCs showed red fluorescence, which indicated that DCs can phagocytose exosomes in a process dependent on temperature, the cytoskeleton and a vacuolar proton ATPase. Next, the authors used the same strategy as the previous work to evaluate if exosomes can fuse with membranes of acceptor cells: exosomes labeled with R-18 were co-cultured with BMDCs, causing an increase in fluorescence as a consequence of the fusion of exosomes with the cells (which entails the mixing of lipids from both membranes and thus the dilution of the dye). Interestingly, the authors assessed the release of exosomal luminal content in the recipient cells by incubating luciferin-loaded exosomes with BMDCs expressing the enzyme luciferase: luciferin was unable to cross the lipid membrane, so the detection of light measured during the assay was due to the oxidation of luciferin transferred from the exosomes to the cytosol, by the resident luciferase enzyme. EM allowed visualizing exosomes in tight contact with the plasma membrane of BMDCs, as well as in the lumen or attached to the phagosomal limiting membrane (although a direct fusion event was not observed).

1.4.2 Functions of exosomes

Elimination of molecules from cells

Over the past 30 years several diverse functions have been attributed to exosomes. Pioneering studies describing the release of the TfR during reticulocyte maturation via exosomes suggested that these membranous structures were used by cells to eliminate obsolete molecules (Harding et al., 1983; Pan et al., 1985), a process essential for

differentiation into erythrocytes. Later studies on reticulocytic cell lines (K562 cells) show that exosomes could also be a means for cells to expel deleterious drugs, such as the release of the DNA-binding anti cancer drug doxorubicin (Chen et al., 2006).

Further reports showed that B cells secrete exosomes that could induce immune responses (Raposo et al., 1996) and that exosomes from B lymphocytes could be targeted to the surface of follicular dendritic cells in germinal centers in the tonsil (Denzer et al., 2000) to transfer MHC class II to these Class II negative cells. Other studies also highlighted that exosomes secreted by intestinal epithelial cells are involved in humoral responses and transfer antigenic information from the apical domain to the immune cells in the gut (Van Niel et al., 2003). Many other studies support the concept that exosomes secreted by one cell may fulfill a function on a recipient cell. The findings that exosomes carry, in addition to proteins and lipids, nucleic acids such as mRNA and microRNA that are functional in recipient cells (Valadi et al., 2007) strengthen this idea. These discoveries altogether inspired the notion that exosomes could in fact shuttle information between cells, and since then countless articles have explored this possibility in diverse models.

Immune responses

Many studies regarding the function of exosomes have focused on the immune system, and were reviewed in several articles (Bobrie et al., 2011; Chaput and Thery, 2011; Thery et al., 2009). DC-derived exosomes may have stimulatory effects on immune cells (potentially useful in terms of cancer immunotherapy, for instance), or cause tolerogenic responses (of interest for the treatment of autoimmune diseases), depending on the maturation state of the secreting cells. Exosomes released by tumor cells carry tumor antigens, and can induce efficient anti-tumor responses if co-administered with adjuvants, or if first administered to DCs, which then present antigens contained in exosomes to T cells (Wolfers et al., 2001).

Cells infected with intracellular pathogens, such as *Mycobacterium tuberculosis* and *Toxoplasma gondii*, have been shown to secrete exosomes that contain microbial components and can promote antigen presentation and macrophage activation, suggesting that exosomes may function in immune surveillance (Schorey and Bhatnagar, 2008).

An interesting study was conducted on pregnancy-associated exosomes (Taylor et al., 2006), and showed that placenta-derived exosomes are present in the sera of pregnant women, at

FIGURE 5 – Roles of tumor-derived exosomes in the promotion of tumors.
Exosomes secreted by cancer cells can use various strategies to create an immunosuppressive microenvironment, generate pre-metastatic niches, and achieve drug resistance, as depicted here, which in turn ensures the sustained growth of the primary tumor and metastases.
From Yang and Robbins, 2011, Clinical and Developmental Immunology

higher levels in those delivering at term. Exosomes isolated from pregnancies delivering at term express higher levels of FasL and MHC class II molecules (HLA-DR) than those delivering preterm, and they are more effective at inhibiting T cell activation in vitro in a FasL-dependent manner, thus suggesting that these vesicles may in fact inhibit maternal immune responses against the fetus.

Recent articles have studied the secretion of exosomes by the eukaryotic intracellular parasite *Leishmania*, and their effect on the host immune system. Exosomes secreted by *Leishmania* can induce a tolerogenic phenotype of macrophages and dendritic cells, which secrete IL-10 at higher levels than TNF-α (Silverman et al., 2010a; Silverman et al., 2010b). These data suggest that exosomes from pathogens may also perform intercellular communication between cells, and modulate the immune system.

Pathology

Exosomes have also been related to disease. Some studies have suggested a direct role of exosomes in the process of pathogenesis, as the release of beta-amyloid peptides associated with exosomes during Alzheimer's disease (Rajendran et al., 2006), or the spreading of the infectious form of the prion protein via exosomes (Coleman et al., 2012; Fevrier et al., 2004; Vella et al., 2007).

Exosomes released by tumor cells, in addition to inducing immune responses directed against the tumor, may also contribute to tumor growth by generating an immunosuppressive microenvironment, establishing a pre-metastatic niche that favors the appearance of metastases, and favoring the development of drug resistance, as shown in **Figure 5** (Yang and Robbins, 2011).

Exosomes as therapeutic agents or diagnostic tools

The discovery that exosomes released by antigen-presenting cells (Raposo et al., 1996; Zitvogel et al., 1998) or tumor cells (Wolfers et al., 2001) could induce efficient immune responses against tumors has lead to their use in clinical trials as anti-tumor immunotherapeutic vectors. Specific purification protocols have been developed so as to isolate clinical grade exosomes under good manufacturing procedures (GMP) (Escudier et al., 2005; Lamparski et al., 2002). The first phase I clinical trial was performed to treat stage III/IV melanoma patients with autologous DC-derived exosomes pulsed with peptides from a

melanoma antigen, MAGE 3 (Escudier et al., 2005). It showed the safety of exosome administration, and an enhanced natural killer (NK) cell effector function in 8/13 patients. Another phase I trial using autologous DC-derived exosomes loaded with MAGE antigens to treat advanced non-small cell lung carcinoma (NSCLC) also demonstrated the safety of the treatment, and an increased NK lytic activity in these patients (Morse et al., 2005). Finally, one phase I trial employed ascites-derived exosomes in combination with granulocyte-monocyte colony-stimulating factor (GM-CSF) for the treatment of advanced colorectal cancer: this treatment was also well tolerated by late-stage cancer patients, and the addition of GM-CSF induced a tumor-specific cytotxic T cell response, as opposed to exosomes injected alone (Dai et al., 2008). The use of DC-derived exosomes, although well tolerated by patients, did not yield proper immunizations in these trials, and thus a study aiming at increasing the immunogenicity of exosomes was performed, and showed that treating DCs with interferon-γ (IFN-γ) lead to the production of exosomes with a higher expression of co-stimulatory and adhesion molecules and a higher capacity to stimulate cytotoxic T cells (Viaud et al., 2011). A phase II clinical trial currently ongoing at Curie and Gustave Roussy Institutes in France to treat NSCLC patients is based on the use of exosomes obtained from autologous DCs treated with IFN-γ and loaded with peptides, combined with cyclophosphamide (Chaput and Thery, 2011), in an attempt to increase the progression-free survival of the patients.

Recently, therapeutic approaches involving exosome-based gene therapy are also being explored, and rely on the safety and lack of toxicity observed in clinical trials (as mentioned above), on the ability of exosomes to cross biological barriers and on their capacity to shuttle functional nucleic acids between cells (O'Loughlin et al., 2012).

Other studies have focused on the molecular characterization of exosomes from biological fluids such as urine and plasma (which are easily obtained with non-invasive methods) in an effort to employ them as tools for the diagnosis and prognosis of several diseases, including cancer (Simpson et al., 2009). More particularly, mRNA and miRNA were found in exosomes (and other vesicles) purified from the serum of patients with glioblastoma (Skog et al., 2008) or ovarian carcinoma (Taylor and Gercel-Taylor, 2008), and thus suggested the use of the nucleic acid content of exosomes as markers of disease progression.

The physiological relevance of exosomes is still unknown today. Most of the studies performed so far have been made using purified and concentrated preparations of vesicles, without any clue as to the proportion of vesicles that are secreted by a given cell type over time, or the concentration of exosomes in a particular medium. In order to convincingly address this issue, tools to specifically inhibit or increase exosome secretion *in vivo* are necessary. This can only be achieved through a detailed knowledge of the intracellular molecules that participate in the biogenesis and the secretion of exosomes. I will now review the current knowledge of these mechanisms.

FIGURE 6 – Mechanisms of endocytosis.
Phagocytosis is the uptake of large particles (including bacteria), and is performed by specialized cells such as phagocytes. Uptake of fluids occurs by a process termed pinocytosis, which includes several mechanisms: macropinocytosis, clathrin-dependent endocytosis, caveolæ-dependent endocytosis, and clathrin- and caveolæ-independent endocytosis. Phagocytosis and macropinocytosis involve the formation of larger vesicles as compared to the other mechanisms, and are dependent on actin-mediated remodeling of the plasma membrane at a large scale. Most internalized cargoes are delivered to the early endosome.
From Mayor and Pagano, 2007, Nature Reviews Molecular Cell Biology

II - Molecular mechanisms of biogenesis and secretion of exosomes

2.1- The endocytic pathway

2.1.1 Different mechanisms of endocytosis

Endocytosis is a process by which cells uptake extracellular materials, such as molecules or particles that cannot enter by diffusion through the hydrophobic plasma membrane. It involves the formation of an intracellular vesicle by invagination and subsequent pinching off of the plasma membrane. This process is crucial to several cellular functions, including nutrient uptake, regulation of the cell surface expression of signaling receptors, remodeling of the plasma membrane, maintenance of cell polarity, antigen presentation and migration. Several mechanisms of endocytosis have been identified (Doherty and McMahon, 2009; Mayor and Pagano, 2007; Nichols and Lippincott-Schwartz, 2001) (**Figure 6**), and can be divided into two main categories according to the nature of the endocytosed material: particles enter cells by phagocytosis (also known as "cell eating"), whereas soluble molecules do so by pinocytosis (also referred to as "cell drinking").

Phagocytosis occurs in specialized cells such as macrophages and other phagocytes, to engulf large particles (including bacteria and other pathogens, and apoptotic cells) in order to destroy them. Binding of the particle to cell surface receptors triggers the remodeling of the plasma membrane (involving actin polymerization) and leads to the formation of pseudopodia that surround the particle and allow its internalization in a structure called phagosome, which is destined to degradation through the action of hydrolytic enzymes contained in lysosomes.

Pinocytosis can occur in all cell types, through several mechanisms: macropinocytosis, clathrin-dependent endocytosis, caveolæ-dependent endocytosis, and clathrin- and caveolæ-dependent endocytosis (**Figure 6**). Macropinocytosis occurs in a similar way as phagocytosis, to the extent that it requires actin remodeling at the plasma membrane to generate lamellipodia, which enclose extracellular fluids and soluble molecules into a large intracellular vesicle. Caveolæ are 50-80 nm flask-shaped invaginations at the plasma membrane, which contain caveolins, a series of proteins with the capacity to bind cholesterol. Clathrin-dependent endocytosis (also referred to as receptor-mediated endocy-

FIGURE 7 – The endocytic pathway.
Internalized cargoes are first directed to early endosomes, where they are sorted towards the degradation or the recycling pathway. The tubular portion of early endosomes gives rise to recycling endosomes by fission; receptors fated for recycling are accumulated here before being directed to the plasma membrane. Conversely, the vesicular element of the early endosome can mature into a multivesicular endosome, where receptors, ligands and other molecules fated for degradation are accumulated in the intraluminal vesicles of these compartments. This compartment can then fuse with lysosomes, which contain hydrolytic enzymes that carry out the degradation of its content. Alternatively, multivesicular endosomes can fuse with the plasma membrane and release the intralumenal vesicles, which are then referred to as exosomes.
From Raposo and Marks, 2007, Nature Reviews Molecular Cell Biology

tosis) has been the most extensively studied mechanism of endocytosis (Benmerah and Lamaze, 2007). It involves the formation of clathrin-coated pits (CCP) by clustering of cargoes via cell surface receptors and polymerization of clathrin lattices through the action of different adaptor complexes. The release of the clathrin-coated vesicle (CCV) from the plasma membrane is mediated by dynamin and requires GTP hydrolysis by this protein.

All these mechanisms lead to the formation of an endocytic vesicle that fuses with an intracellular compartment known as early endosome.

2.1.2 Intracellular compartments of the endocytic pathway

The endocytic pathway consists of a series of highly dynamic membrane compartments, which lead to the recycling of endocytosed materials back to the plasma membrane, or their degradation (Gruenberg, 2001; Gruenberg and Stenmark, 2004; Stenmark, 2009; Woodman and Futter, 2008) (**Figure 7**). These compartments are enriched in specific sets of proteins (Williams and Urbe, 2007) (**Figure 8**) including Rab GTPases (Stenmark, 2009), and they can be identified by the rate at which they are accessed by endocytic tracers or by ligands of cell surface receptors, and by morphological characteristics. Imaging methods from optical to electron microscopy, new methods such as high resolution live cell microscopy, and novel developments in EM such as high pressure freezing and electron tomography have been an asset to further enlighten the biogenesis, morphology and dynamics of these compartments.

Early endosomes are tubulo-vesicular structures located at the periphery of the cells, where efficient sorting of the endocytosed material occurs. They are identified by the presence of internalized receptors such as TfR 5-10 min following internalization, and the markers early endosome antigen 1 (EEA1), Rab4 and Rab5. The mildly acidic pH of these endosomes induces the uncoupling of ligands, which will be directed toward the degradation pathway, and their receptor, which can be recycled. Receptors that are fated for recycling accumulate in tubular structures named recycling endosomes, which are less acidic than early endosomes and are located in the perinuclear region, near the Golgi apparatus. They are reached by TfR after 15 min of endocytosis, and are marked by the presence of Rab4 ad Rab11.

FIGURE 8 – Changes in protein and lipid content among compartments of the endocytic pathway.

The abundance of certain proteins and lipids varies during the maturation from early to late endosomes, as well as in lysosomes.

From Williams and Urbé, 2007, Nature Reviews Molecular Cell Biology

Late endosomes or MVEs are formed by the maturation of early endosomes (Stoorvogel et al., 1991). They are characterized by the presence of ILVs, which are produced by inward budding of the limiting membrane of the vesicular domains of the early endosome (tubular elements giving rise to the recycling endosome). They accumulate, on the ILVs, cell-surface receptors and other molecules fated for degradation, such as the epidermal growth factor receptor (EGFR), which reaches the compartment 30 min after endocytosis. Markers of these compartments include CD63, Rab7, and lysosomal-associated membrane proteins (LAMP). Their main fate is to fuse with lysosomes that contain lysosomal hydrolases, thus ensuring the degradation of their content. Lysosomes are highly acidic compartments with a characteristic electron-dense morphology, which possess markers such as LAMP-1 and LAMP-2, CD63, and Rab9.

However, as detailed in section *1.2.1 Discovery of exosomes*, several research groups have demonstrated that compartments with hallmarks of MVEs are fated for fusion with the plasma membrane leading to the exocytosis of the ILVs as exosomes, rather than for fusion with lysosomes. This has prompted the question of whether one unique type of MVE could have different fates, or if different subpopulations of MVEs occur simultaneously in cells, some being destined to the degradation pathway whereas others are fated for exocytosis.

2.1.3 Different types of MVEs coexist in cells

Several lines of evidence have demonstrated the existence of different populations of MVEs. With the use of a newly set up electron microscopy technique allowing the staining of cholesterol with perfringolysin O (derived from a cholesterol-binding toxin) in cryosections (Mobius et al., 2002), Mobius and colleagues studied the distribution of cholesterol in the different compartments of the endocytic pathway (Mobius et al., 2003). The authors observed that in EBV-transformed B cell lines cholesterol-positive and -negative MVEs coexisted. MVEs positive for the lipid were the main cholesterol-containing endocytic compartments, and the majority of the staining was located on the ILVs of these compartments, in agreement with articles describing the lipid composition of exosomes, which show the clear enrichment of exosomes in cholesterol as compared to other cellular membranes. A fusion profile showing the release of cholesterol-enriched vesicles (exosomes) was also seen in these cells.

Additionally, a study of the effect of epidermal growth factor (EGF) stimulation on the formation of MVEs (White et al., 2006) showed the existence of two distinct populations of MVEs: after internalization the EGFR traffics to a subpopulation of CD63-positive endosomes, and a detailed immuno-EM analysis showed the presence of another subset of MVEs containing LBPA and CD63, but negative for EGFR. It is likely that the MVEs containing LBPA are fated for degradation more than fusion with the plasma membrane, since exosomes do not contain LBPA as evidenced by studies performed in B cells (Wubbolts et al., 2003).

More recently, the comparison of immature DCs and DCs undergoing cognate interactions with T cells (i.e. cells loaded with an antigen and incubated with antigen-specific T cells) revealed the presence of different MVEs in these cells (Buschow et al., 2009). In immature cells, MHC class II molecules are sorted into MVEs mainly fated for degradation. In the presence of antigen-specific T cells, DCs are capable of secreting exosomes enriched in MHC class II and the tetraspanin CD9, from newly formed MVEs carrying both proteins. This points to a difference in the mechanism of MHC class II sorting in these two cells: in immature DCs it involves the ubiquitination of MHC class II molecules in order to be incorporated into ILVs of endosomes fated for lysosomal degradation. The other mechanism occurs in DCs that undergo cognate interactions with T cells: loading of MHC class II together with CD9 on ILVs destined to be released as exosomes is independent of ubiquitination, but instead relies on the incorporation of MHC class II in detergent-resistant membranes containing CD9.

Interestingly, the description of exosomes secreted by epithelial intestinal cells has demonstrated that exosomes secreted by the apical and the basolateral membranes are different (van Niel et al., 2001). Western blotting analysis as well as mass spectrometry experiments performed on exosomes purified from the apical or the basolateral sides showed that both types of exosomes share markers such as MHC class I and II, and the tetraspanin CD63 among others, but they differ in the content of other proteins, such as syntaxin 3 in apical exosomes and A33 in basolateral exosomes. The existence of different subpopulations of exosomes secreted by the same cell suggests that they derive from different types of MVEs, thus supporting the hypothesis that different types of MVEs can coexist in the same cell.

FIGURE 9 – The ESCRT pathway.
Schematic representation of the four ESCRT complexes, and the domains through which they interact with the endosomal membrane and cargo proteins, as well as with the other complexes.
From Teis et al., 2009, Cell

2.2- Biogenesis of exosomes

2.2.1 ESCRT-dependent mechanisms

As stated before, exosomes are formed within endocytic compartments during the maturation of endosomes. Insights into the molecular mechanisms that lead to the formation of ILVs emerged with the discovery of genetic mutants in the yeast *Saccharomyces cerevisiae*, a model organism that has an analogous pathway of lysosomal degradation that involves trafficking to the vacuole. Mutant strains for vacuolar sorting (Vps) showed an impaired MVE formation, and the accumulation of cargoes fated for vacuolar degradation in abnormal compartments next to the vacuole, the class E compartments (Odorizzi et al., 1998; Raymond et al., 1992; Rieder et al., 1996). Further research showed that these proteins, and their orthologues in mammalian cells, could form complexes named ESCRT (for "Endosomal Sorting Complexes Required for Transport", **Figure 9**), which were described in a rapid succession of articles: ESCRT-I (Katzmann et al., 2001), ESCRT-II (Babst et al., 2002b) and ESCRT-III (Babst et al., 2002a). Another complex, nowadays known as ESCRT-0, which functions upstream of ESCRT-I, was also described (Katzmann et al., 2003). These complexes can interact with each other and are recruited to the endosomal limiting membrane where they carry out their function. The recruitment of these protein complexes to the endosome membrane is transient, since the accessory AAA+ATPase VPS4 is responsible for the release of the complexes once their function is complete (Babst et al., 1998), so that the machinery can be recycled to other sites. Interestingly, some of these proteins have also been linked to the budding of enveloped viruses, as well as in the process of abscission during cytokinesis (**Figure 10**). The organization and function of each ESCRT complex will now be described in more detail (Hanson and Cashikar, 2012; Hurley, 2010; Williams and Urbe, 2007, **Figure 11**).

ESCRT-0

The ESCRT-0 complex is a heterodimer formed by HRS (HGS, hepatocyte growth factor regulated kinase substrate, Vps27 in yeast) and STAM (signal transducing adaptor molecule, in humans two isoforms exist: STAM1 and STAM2; Hse1 in yeast). This complex is essential for the initial selection of ubiquitylated cargo at the endosomal membrane. HRS binds phos-

FIGURE 10 - Involvement of ESCRT proteins in different cellular processes.
ESCRT proteins can participate in multivesicular endosome biogenesis, cytokinesis, viral budding and autophagy. In MVE biogenesis, all four ESCRT complexes are recruited sequentially to the endosome limiting membrane to carry out their function. In cytokinesis, the ESCRT-III complex is recruited by the midbody protein CEP55 and ALIX, as well as the ESCRT-I complex. During viral budding, ESCRT-III, VPS4, ESCRT-I and ALIX are required for viral budding.
From Hurley and Hanson, 2010, Nature Reviews Molecular Cell Biology

phatidylinositol-3-phosphate (PI(3)P), a highly abundant phospholipid in endosome membranes, through its FYVE domain (Raiborg et al., 2001), thus ensuring the recruitment of the complex to the endosomal membrane. Both HRS and STAM contribute to cargo selection by interacting with monoubiquitinated proteins through their ubiquitin binding domains (UBDs) (Urbe et al., 2003). Both subunits can also recruit clathrin, which forms a flat double-layered coat (Raposo et al., 2001; Sachse et al., 2002) on the endosomal membrane where HRS is accumulated, and which is thought to concentrate cargo into microdomains that will subsequently bud to form ILVS. Hrs recruits ESCRT-I by direct interaction with the ESCRT-I subunit TSG101 (Bache et al., 2003; Katzmann et al., 2003).

ESCRT-I

The human ESCRT-I complex consists of TSG101 (Vps23 in yeast), VPS28 (Vps28 in yeast), one of four isoforms of VPS37 (mammalian VPS37A-VPS37D, Vps37 in yeast), and one of three MVB12 subunits (mammalian MVB12A/B and UBAP1, Mvb12 in yeast). ESCRT-I can bind ubiquitin through the UBD of TSG101 (Teo et al., 2004), which increases the efficacy of ubiquitinated cargo sorting. In addition to ESCRT-0 and ubiquitin, the complex has been shown to interact with ALIX, and ESCRT-II in yeast. TSG101 is essential to all ESCRT-dependent pathways, and it is interesting to note that ESCRT-I subunits are mutually dependent on each other for stability, and thus TSG101 depletion leads to a loss of function of the entire complex (Hanson and Cashikar, 2012).

ESCRT-II

The ESCRT-II complex is a heterotetramer consisting of one subunit of VPS22/SNF8 (Vps22 in yeast) and VPS36 (Vps36 in yeast), together with two subunits of VPS25 (Vps25 in yeast). In yeast, Vps36 contains a GLUE domain, which allows interaction with Pi(3)P, ubiquitin and ESCRT-I, and the two copies of Vps25 are able to bind the ESCRT-III subunit Vps20 (Teis et al., 2010). Thus, in yeast the ESCRT-II complex facilitates the recruitment of ESCRT-III to endosomal membranes (Babst et al., 2002b). In mammalian cells, however, the inactivation of ESCRT-II did not affect viral budding (Langelier et al., 2006), and this complex does not seem to be involved in cytokinesis (Carlton and Martin-Serrano, 2007) or EGFR degradation (Bowers et al., 2006), thus suggesting that other mediators might be responsible for the recruitment of ESCRT-III.

FIGURE 11 – The ESCRT machinery.
The ESCRT machinery consists of four complexes (and accessory proteins) that interact with each other and are recruited sequentially and transiently to the limiting membrane of endosomal compartments. They were initially described as the machinery involved in the sorting of ubiquitinated proteins into the intralumenal vesicles, and the formation of these intralumenal vesicles. ESCRT-0 is responsible for the clustering of ubiquitinated cargoes in domains of the limiting membrane. ESCRT-I and -II are thought to drive the deformation of the membrane into buds, and ESCRT-III is required to perform the scission of the bud into the lumen of the compartment. The ATPase complex is necessary to release ESCRT-III components, thus allowing this process to occur elsewhere.
From Williams and Urbé, 2007, Nature Reviews Molecular Cell Biology

ESCRT-III

In yeast, the ESCRT-III complex consists of four core subunits: Vps20, Vps32/Snf7, Vps24 and Vps2, and three associated or regulatory proteins: Did2, Vps60 and Ist1 (increased sodium tolerance protein 1). In mammalian cells, ESCRT-III proteins are also known as charged multivesicular body proteins (CHMPs), and include 12 proteins, some of which are multiple homologs of yeast components (see **Table 2**). The core subunits are then CHMP6, CHMP4, CHMP3 and CHMP2 in mammalian cells, and the associated proteins CHMP1, CHMP5 and IST1. ESCRT-III differs from the other stable, cytoplasmic complexes, in that its components are soluble monomeric proteins that upon recruitment to the endosomal membrane by upstream pathway components can polymerize into filaments (Babst et al., 2002a). In yeast, the ESCRT-II protein Vps25 binds to Vps20 (Teis et al., 2010), initiating ESCRT-III recruitment to the endosome and complex formation. Vps20 then recruits Vps32/Snf7 (CHMP4 in mammalian cells), which polymerizes and forms filaments involved in the clustering of cargo (Teis et al., 2008). Vps32 recruits Vps24, which is proposed as a capping protein (Saksena et al., 2009), and in turn Vps24 recruits Vps2, which engages the AAA-ATPase Vps4 that dissociates ESCRT-III complexes. Vps32 also recruits the accessory protein Bro1/Alix, which stabilizes Vps32 filaments and recruits the deubiquitinating enzyme Doa4 necessary for cargo deubiquitination (Hurley, 2010). ESCRT-III related proteins CHMP1, CHMP5 and IST1 have been described as modulators of Vps4 ATPase activity (Nickerson et al., 2010).

Recently, the ESCRT-III complex has been linked to the scission of buds induced by ESCRT-I and II to form ILVs in a cell-free reconstitution of MVE biogenesis with the use of giant unilamellar vesicles (GUVs) (Wollert and Hurley, 2010). Addition of Vps20, Vps32 and Vps24 to ESCRT-I and -II containing GUVs induced the localization of Vps20 and Vps32 to bud necks, and the efficient detachment of ILVs, indicating that scission of the buds was occurring.

Accessory proteins

VPS4 (Vps4 in yeast) is an essential element of the ESCRT pathway, since it catalyzes the dissociation of ESCRT-III subunits from the endosome limiting membrane, allowing these components to recycle for subsequent rounds of activity. This AAA+ATPase was the first class E protein with a known function (Babst et al., 1998), and it is the only energy-requiring component of this pathway. In yeast, depletion of Vps4 induces the accumulation of ESCRT

Table 2 – ESCRT complex proteins.
Here are listed the different subunits of each ESCRT complex, in yeast and human (along with the alternate names that have been used).
From Hanson and Cashikar, 2012, Annual Reviews of Cell and Developmental Biology

components on endosome membranes with a class E phenotype. In mammalian cells, a dominant negative form of this protein with an impaired capacity to hydrolyze ATP inhibits the formation of ILVs and promotes accumulation of ESCRT proteins on aberrant endosomes (Sachse et al., 2004). In humans, two isoforms of VPS4 (A and B) exist, and are 80% identical. VPS4 assembles into a ring-shaped oligomer in association with its cofactor VTA1 (LIP5), and disassembles ESCRT-III by pulling each subunit into its central pore for partial or complete unfolding, and then releasing them as soluble monomeric proteins (Hanson and Cashikar, 2012).

The accessory protein ALIX (ALG-2 interacting protein X, Bro1 in yeast) has been shown to interact with ESCRT-I and ESCRT-III (Strack et al., 2003). It has also been shown to be a key mediator in viral budding (Fisher et al., 2007) and cytokinesis (Carlton and Martin-Serrano, 2007). Nevertheless, ALIX was first described as a potential regulator downstream of ALG-2, a protein required for apoptosis (Missotten et al., 1999); which was later confirmed by an article showing that the complex ALIX/ALG-2 is a regulator of cell death, controlling caspase-dependent and -independent pathways (Trioulier et al., 2004). ALIX is a multifunctional protein (Sadoul, 2006), which appears to play a key role in MVE formation also. During MVE formation, ALIX binds CHMP4 and TSG101, as well as other proteins, but its exact function in the ESCRT pathway for ILV formation is unknown. Data suggest that this protein might be responsible for the recruitment of ESCRT-III components to ESCRT-I domains on the endosome membrane. Alternatively, ALIX could activate CHMP4 to favor ESCRT-II assembly, as shown by expression of the yeast Bro1 domain, which stabilizes ESCRT-III (Wemmer et al., 2011).

ESCRTs and exosomes

A role for ESCRT proteins in exosome biogenesis has been proposed based on several lines of evidence. Exosomes from DCs and B cells have been shown to contain ubiquitinated proteins (Buschow et al., 2005), and monoubiquitination is a signal for the targeting of proteins to ILVs during MVE maturation (see above). Nonetheless, the treatment of these exosomes to release all proteins that are not integrated in the membrane indicated that these ubiquitinated proteins are soluble and not inserted in the exosome membrane, thus implying that their presence in the exosomes is not a consequence of ESCRT function.

However, proteomic studies have shown the presence, in exosomes from different cell types, of ESCRT proteins such as TSG101 and ALIX (even before they were described as part of the ESCRT complex, see Thery et al., 2001), suggesting that exosome biogenesis could be achieved through an ESCRT-dependent mechanism.

A study performed on mouse DCs has proposed a role of HRS in the release of exosomes (Tamai et al., 2010). The authors detected a significant decrease in exosome release by HRS-deficient DCs (siRNA-treated DCs, or cells isolated from HRS knock-out mice) stimulated for exosome secretion by treatments with OVA or a calcium ionophore. The impaired exosome secretion resulted in a defect in antigen presentation to cytotoxic T cells, and this was not due to a compromised lysosomal degradation of OVA.

Interestingly, ALIX has been linked to exosome biogenesis in various studies. Géminard and colleagues (Geminard et al., 2004) observed that during reticulocyte maturation the chaperone hsc70 binds TfR through its cytoplasmic domain upon adaptor protein 2 (AP2) degradation by the proteasome. Hsc70 binding decreases TfR receptor targeting to exosomes, and thus the authors hypothesized that another molecule was responsible for this process. Binding of ALIX to the same region in the cytoplasmic domain of TfR in the absence of AP2 suggested that ALIX links the TfR to the ESCRT machinery for sorting onto ILVs. A more recent study also suggests a role of ALIX in the biogenesis of exosomes (Baietti et al., 2012). Exosomes were shown to contain syndecan -ubiquitous transmembrane proteins that bind heparan sulphate from the extracellular matrix-, syntenin -a small PDZ scaffolding protein that can bind syndecans via their cytoplasmic domain- and ALIX, which in turn interacts with syntenin. Overexpression of syntenin induced an increase in the release of exosomes (as evidenced by an increase in exosomal markers CD63 and HSP70), which was dependent on ALIX. Conversely, downregulation of syndecan, syntenin or ALIX impaired exosome release. Next, the authors showed that budding of endosomal membrane domains containing CD63 and syndecan was induced by syntenin. The biogenesis of syndecan, syntenin and ALIX-containing exosomes was dependent on ESCRT-II, ESCRT-III and VPS4 function, as well as on clustering of syndecan. These data support a role of Alix in exosome biogenesis and exosomal sorting of syndecans via an interaction with syntenin.

2.2.2 ESCRT-independent mechanisms

The existence of different MVEs in a single cell (as mentioned above) has hinted at the possibility that different mechanisms operate in cells to create these different compartments, thus challenging the notion that the ESCRT machinery always mediates MVE biogenesis.

Stuffers and collaborators used an interesting approach to address the question of whether ESCRTs are necessary for ILV biogenesis. They reasoned that the lack of total MVE inhibition upon ESCRT inactivation observed in several studies could be due to an incomplete depletion of ESCRT subunits, or to the existence of ESCRT-independent mechanisms of ILV budding. Therefore, they simultaneously inactivated four ESCRT proteins (Hrs, Tsg101, Vps22 and Vps24), so as to target all four ESCRT complexes and completely abolish ESCRT function (Stuffers et al., 2009). Cells inhibited for all four ESCRTs showed an important alteration of the endocytic compartments, and the presence of enlarged MVEs with few internal vesicles next to the limiting membrane. However, ILVs did form in these aberrant structures, indicating that mechanisms other than the ESCRT pathway can ensure the formation of MVEs.

The group of M. Simons proposed a pathway for the budding of vesicles into MVEs that was independent of ESCRT function, and required the sphingolipid ceramide (Trajkovic et al., 2008). They performed their study in an oligodendrial cell line expressing the proteolipid protein (PLP), which they showed was released associated with exosomes. Next, by making cells overexpress a GTPase-deficient form of Rab5 (Rab5^{Q79L}) that induces the fusion of early endosomes (Stenmark et al., 1994), the authors saw by confocal microscopy that PLP co-localized with markers of lipid rafts (flotillin and glycosylphosphatidylinositol), and not with Hrs or EGFR, suggesting different mechanisms of budding into ILVs for PLP and EGFR. This was analyzed by depletion of different ESCRT proteins: whereas Hrs and Tsg101 inactivation reduced the sorting of EGFR into ILVs, it had no effect on the budding of PLP into these vesicles. Furthermore, inactivation of Tsg101 and Alix, and a dominant negative form of Vps4 did not impair secretion of PLP-bearing exosomes, suggesting that the endosomal trafficking of PLP was independent of ESCRT function. The finding that exosomes were enriched in ceramide (a sphingolipid formed by hydrolysis of sphingomyelin by the enzyme neutral sphyngomyelinase or nSMase) prompted the authors to analyze its role in exosome

biogenesis: treatment of cells with nSMase inhibitors decreased exosome release (as evidenced by a decrease of PLP in the exosome fraction), and budding of PLP into the lumen of endosomes. GUVs were then prepared with fluorescently labeled lipids that mimicked liquid-disordered and liquid-ordered (i.e. raft-like) lipid phases, and addition of SMase induced the budding of the liquid-ordered lipid phase into the lumen of the vesicles. These data thus suggested that PLP sorting into ILVs occurred via an ESCRT-independent pathway that required lipid rafts and the presence of ceramide.

Studies in melanocytic cell lines have also suggested the existence ESCRT-independent mechanisms for MVE formation. The first evidence came from a study of the sorting into ILVs of PMEL17 (PMEL) (Theos et al., 2006), a component of melanosomes (lysosome-related organelles of melanocytes, which derive from MVEs). Inhibition of ESCRT function by overexpression or inactivation of HRS, TSG101, or the ATP-binding defective form of VPS4 VPS4(K173A), or inhibition of the ubiquitylation of PMEL, did not alter the sorting of PMEL onto ILVs; a lumenal domain from the PMEL protein was shown to be required. Furthermore, MVEs were still able to form in cells where the ESCRT machinery had been disrupted, albeit their morphology was different from that of control cells, suggesting that MVEs may be created by ESCRT-dependent and -independent mechanisms, giving rise to different populations of MVEs. A more recent study has proposed a role of the tetraspanin CD63 in the sorting of the lumenal domain of PMEL to ILVs (van Niel et al., 2011). In MNT-1 melanoma cells, CD63 is detected together with PMEL in MVEs, more abundantly on ILVs than on the limiting membrane, and their sorting is independent of the ESCRT-I protein TSG101. Inactivation of CD63 inhibits formation of ILVs in endosomes, and causes PMEL to accumulate at the limiting membrane, thus showing that CD63 participates in the sorting of this protein to ILVs. Inactivation of TSG101 decreases degradation of the remaining transmembrane fragment of PMEL (CTF), which remains located at the limiting membrane of endosomes and is not targeted for lysosomes. The results show that sorting of the luminal domain of PMEL requires CD63 but not a functional ESCRT machinery, whereas the disposal of the transmembrane domain has opposite requirements. Interestingly, in the absence of CD63 the lumenal domain is targeted for ESCRT-dependent degradation together with the transmembrane fragment, revealing tight interplay between ESCRT-dependent and -independent sorting mechanisms for a single cargo.

2.2.3 Other mechanisms

Several other intracellular effectors have been proposed as mediators of exosome biogenesis.

While studying the effect of citron kinase, a RhoA (Ras homolog gene family, member A, a small GTPase) effector, on HIV-1 viral release in human cell lines, Loomis et al discovered that in addition to increasing viral production and release (as evidenced by the increased recovery of p24, the capsid protein of the virion, in the cell-conditioned medium) citron kinase enhanced the secretion of vesicles bearing proteins which are generally enriched in exosomes (Hsc70, CD82, LAMP-1) (Loomis et al., 2006). This suggests that citron kinase is involved in the exocytosis of late endosomal compartments.

T cells have been shown to secrete vesicles that carry an unprocessed form of the pro-apoptotic protein Fas ligand (FasL) upon activation (Martinez-Lorenzo et al., 1999). Diacyl glycerol kinase α (DGKα), a kinase highly expressed in T lymphocytes, inhibits activation-induced secretion of exosomes bearing FasL when overexpressed (Alonso et al., 2007); the exosomes were identified based on the presence of CD63 and LAMP-1, and the absence of plasma membrane markers CD45 and CD28. On the contrary, when DGKα was inhibited, the secretion of these exosomes was increased, having an impact on activation-induced cell death and suggesting a role of DGKα in the regulation of exosome secretion or MVE biogenesis. The observation that DGKα inhibition also increased the presence of CD63-positive compartments in these cells seemed to favor the second hypothesis (Alonso et al., 2007). Further analysis appeared to confirm DGKα as a negative regulator of the formation of mature MVEs (Alonso et al., 2011), which explains the decrease in exosome secretion, although the authors use LBPA as a marker of MVEs and as mentioned before (see *2.1.3 Different types of MVEs coexist in cells*) LBPA has been linked to MVEs fated for lysosomal degradation and not fusion with the plasma membrane resulting in exosome release.

Another study has proposed the involvement of BIG2 in the release of exosome-like vesicles bearing TNFR1 (Islam et al., 2007). Vesicle formation, a process that would account for TNFR1 trafficking to the plasma membrane prior to its release, is controlled by ADP-ribosylation factors (ARFs), which are activated by guanine nucleotide- exchange factors that catalyze replacement of GDP by GTP. The brefeldin A (BFA)-inhibited guanine

nucleotide-exchange proteins BIG1 and BIG2 were thus studied in the context of the release of TNFR1 by HUVEC cells, and BIG2 emerged as a mediator of exosome-like vesicle release. However, these results must be interpreted with caution, since the authors have measured the release of TNFR1 by ELISA as a means of detecting the release of vesicles, without actually confirming the presence of vesicles with hallmarks of exosomes in the cell-conditioned medium.

Interestingly, phosphatidyl-inositol 3 (PI(3)) kinase was revealed as a mediator of multivesicular body morphogenesis. Treatment with wortmannin (an inhibitor of PI(3)-kinase) induced enlarged vacuoles in melanoma cells, which contain few ILVs that do not fully detach from the limiting membrane (Fernandez-Borja et al., 1999). This role of PI(3)-kinase, however, was not directly linked to exosome biogenesis.

Aggregation of molecules at the plasma membrane has also been described as a sorting signal to target proteins to the exosome secretion pathway. The lipid analog N-Rh-PE accumulates in small clusters at the plasma membrane of reticulocytes, and has been shown to accumulate in exosomes: this is not the case for another lipid analog C6-NBD-SM, which does not form clusters and is recycled to the plasma membrane. Also, cross-linking of surface proteins (TfR and AChE) with specific antibodies, which induces clusters of these proteins at the plasma membrane, induced an increase of their release with exosomes (Vidal et al., 1997). A similar mechanism of protein targeting was described in Jurkat T cells, where clustering of CD43 on the cell surface via specific antibodies induced an increased amount of CD43 in exosomes, without seemingly altering the total exosome yield (Fang et al., 2007).

2.3- Secretion of exosomes

The secretion of exosomes involves the transport of the mature MVE to the vicinity of the plasma membrane for docking and fusion, and consequently the release of the ILVs. This process must encompass a large variety of molecules responsible for the different steps: proteins of the cytoskeleton (actin, tubulin, etc) and molecular motors (kinesins) probably mediate the transport to the plasma membrane, while several other families of proteins including molecular organizers of membrane trafficking (Rab proteins) and the fusion

machinery (SNAREs) are likely necessary for the final phases of fusion. The molecular machinery in charge of exosome secretion has yet to be deciphered, but some recent advances have been made in its description and will be discussed in this section.

2.3.1 Constitutive secretion or regulated secretion?

Most studies presented so far have analyzed the constitutive secretion of exosomes by different cell types. However, the induction of exosome secretion has been described in several models. In the hematopoietic cell line K562, a rise in intracellular calcium levels induces exosome release (Savina et al., 2003). Degranulation of mast cells has also been related to an increase in exosome release (Laulagnier et al., 2004a; Raposo et al., 1997). T-cell receptor (TCR) activation induces the secretion of exosomes bearing the TCR/CD3/ζ complex, unlike the activation of T cells via PMA/ionomycin treatment (Blanchard et al., 2002). Alternatively, exosome secretion by murine DCs may be increased by cognate interactions with antigen-specific CD4+ T lymphocytes (Buschow et al., 2009). The secretion of exosomes by rat cortical neurons can be stimulated by depolarization of the cells (Faure et al., 2006). Moreover, γ-irradiation-induced DNA damage can promote exosome secretion through the activation of the p53-regulated protein TSAP6 (Lespagnol et al., 2008; Yu et al., 2006).

2.3.2 RAB proteins

Rab proteins are the largest family of small monomeric GTPases (including over 60 members in humans), and they are distributed in distinct intracellular compartments so as to carry out their function as major regulators of vesicular transport between organelles (reviewed in (Stenmark, 2009; Zerial and McBride, 2001) (**Figure 12**). The regulatory function of these proteins is based on their capacity to switch from a GDP bound form ("inactive") to a GTP bound form, which is considered to be the active conformation. This cycle is regulated, among other effectors, by guanine nucleotide exchange factors (GEFs), which facilitate GDP released and subsequent binding of GTP, and by GTP hydrolysis, which can be catalyzed by the GTPase activity of the Rab protein itself or by a GTPase-activating proteins (GAPs). Effector molecules are activated by the active GTP-bound Rab.

FIGURE 12 – Location of Rab proteins in intracellular compartments.
Rab proteins are located in particular intracellular compartments as depicted here, which allows them to carry out their function as regulators of intracellular transport. Note that several Rab proteins may be found in a single compartment, although on separate membrane microdomains (referred to as the Rab domains).
From Stenmark, 2009, Nature Reviews Molecular Cell Biology

Rab proteins can regulate intracellular transport of vesicles by acting at different stages of this process: they have been implicated in vesicle budding, motility through the interaction of these vesicles with the cytoskeleton, and tethering which will ultimately result in the fusion with the target compartment. Interestingly, different Rab proteins can be found on the membrane of the same organelle, albeit on different membrane microdomains, referred to as Rab domains. Such is the case of early and recycling endosomes, which present multiple combinations of RAB4, RAB5 and RAB11 domains on their surface. Rab proteins specify membrane identity by recruiting specific downstream effectors to particular membrane microdomains.

The first evidence for the involvement of Rab proteins in exosome secretion came from studies on the erythroleukemia cell line K562, which, like reticulocytes, release TfR- bearing exosomes. Overexpression of a dominant negative form of Rab11 (unable to hydrolyze GTP) induced a decrease in exosome secretion, evidencing a role for RAB11 in exosome secretion (Savina et al., 2002). Further studies suggested that RAB11 was involved in the tethering of MVEs to the plasma membrane, the final fusion step requiring the presence of calcium (Savina et al., 2005).

A screening performed by our group in HeLa-CIITA cells provided further insights into the role of Rab GTPases in exosome secretion (Ostrowski et al., 2010). Individual Rab components (59 genes in total) were targeted by an RNA interference approach, and the measure of exosome secretion in the cell-conditioned medium indicated that some of these proteins could modulate this process, such as RAB2b, RAB5a, RAB9a, RAB27a and RAB27b. However, Rab proteins identified in other models (see in this section: RAB11a, RAB35, or RAB7 which is associated to late endosomes) did not appear to be involved in exosome secretion by HeLa cells in this screen. Additional experiments to unravel RAB27a and b function confirmed the decrease in exosome secretion upon Rab inactivation, and showed that these two isoforms have different roles in exosome release: silencing of RAB27a induced an increase in MVE size and altered its docking to the plasma membrane, whereas RAB27b invalidation altered the docking to the plasma membrane, but also caused smaller MVEs to accumulate in the perinuclear region.

Another screen was performed that targeted Rab-activating proteins (Rab-GAPs) in a murine oligodendroglial cell line (Hsu et al., 2010), revealing TBC1D10A-C as regulators of exosome

secretion. Furthermore, Rab35 was identified as the target of TBC1D10A-C, and the inhibition of Rab35 function (either by depletion with an siRNA, or by the use of a dominant negative mutant) induced a significant decrease in the secretion of PLP-bearing exosomes. The authors suggest that Rab35 regulates exosome secretion by controlling the docking of MVEs with the plasma membrane.

2.3.3 SNARE proteins

During intracellular vesicle trafficking, the final step fusion of the vesicle with the acceptor membrane depends on a family of molecules named SNAREs (for soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptors, reviewed in Jahn and Scheller, 2006; Proux-Gillardeaux et al., 2005; Zylbersztejn and Galli, 2011). SNARE proteins were originally categorized as vesicular SNARES (v-SNARES, also known as VAMP for vesicular-associated membrane proteins) if located on the membrane of vesicles, and target SNAREs (t-SNAREs) if found on the membrane of acceptor compartments. A more recent nomenclature considers the structural features of SNARE proteins and divides them into R-SNARES and Q-SNARES -depending on the type of residue found in the SNARE motif of each protein, an arginine (R) or a glutamine (Q). SNARE proteins from opposing compartments combine to form a four-α helix bundle, which leads to a tight connection of the membranes of both donor and acceptor compartments, and initiates the membrane fusion (**Figure 13**). The recycling of SNAREs is achieved through the dissociation of the helical bundle, which is mediated by the AAA+ ATPase NSF (N-ethylmaleimide-sensitive factor).

The SNARE complex that would be involved in the fusion of MVEs with the plasma membrane to release exosomes has not been extensively studied. A publication from the group of M.I. Colombo has hinted at a role of VAMP7 and the NSF ATPase (necessary for SNARE disassembly) in the exocytosis of MVEs to release acetylcholinesterase (AChE)-containing exosomes in the human leukemia K562 cell line (Fader et al., 2009), but further studies need to be performed in other cell lines to confirm/extend these results.

FIGURE 13 – Formation of the SNARE complex during vesicle docking and fusion.
SNARE proteins located on the vesicle membrane and the acceptor membrane combine to form a SNARE complex, which by proceeding from a loose to a tight conformation allows the fusion of both membranes. The remaining SNARE complex is recycled by dissociation of its components, through the activity of the NSF ATPase.
From Jahn and Scheller, 2006, Nature Reviews Molecular Cell Biology

2.3.4 Other mechanisms

Genetic approaches in the worm *Caenorhabditis elegans* have proposed the V0 subunit of V-ATPase as a possible mediator of the fusion of MVEs with the plasma membrane. Electron microscopy observation of the worms showed that cuticle mutants appeared to specifically accumulate MVEs that didn't fuse with the plasma membrane (Liegeois et al., 2006). The V0 subunit was identified as the mutated gene responsible for this phenotype, suggesting that the V-ATPase is required for the secretion of MVEs containing 50-100 nm diameter ILVs at the apical epidermal plasma membrane, leading to the production of the cuticle. However, it is still unclear whether the V0 ATPase is involved in exosome secretion in mammalian cells because there are several isoforms, which renders inactivation experiments difficult to interpret.

The effect of TSAP6 (STEAP3) on exosome secretion was also analyzed in different studies. Transcriptionally controlled tumor protein (TCTP) is not secreted through a classical pathway. The p53-inducible 5-6 transmembrane protein TSAP6 directly interacts with TCTP, and the overexpression of TSAP6 leads to an increased secretion of TCTP, which occurs through the release of exosomes bearing TCTP to the extracellular space (Amzallag et al., 2004). The analysis of p53 function after γ-radiation of two different lung cancer cell lines (with a wild-type or mutated p53) further extended these results. γ-irradiated cells with a functional p53 gene showed an increased exosome secretion, and TSAP6 (a p53-inducible protein) was involved in this process (Yu et al., 2006). Also, BMDCs from TSAP6-deficient mice secreted less exosomes than control cells, and exosome secretion induced by p53 in DNA damaging conditions was abrogated in TSAP6-null cells from different origins (dendritic cells, embryo fibroblasts and spleen cells) (Lespagnol et al., 2008).

The activity of phospholipase D (PLD), an enzyme that hydrolyzes phosphatidyl-choline to generate choline and phosphatidic acid (PA), may also be important for exosome release (Laulagnier et al., 2004a). Exosomes secreted by mast cells are enriched in an active PLD2 isoform, and the overexpression of an active PLD2 in these cells induces an increase in exosome release upon degranulation.

Most of the topics described in this Introduction, and especially the relation between exosomes and the immune system, were the subject of a recent review from our group entitled "Exosome secretion: molecular mechanisms and roles in immune responses (Article 1), published in Traffic.

Article 1 – Exosome secretion: molecular mechanisms and roles in immune responses

OBJECTIVES

The main objective of my thesis has been to gain insights on the molecular mechanisms that drive exosome biogenesis and secretion. I especially focused on three families of proteins: ESCRTs, Rabs, and SNAREs. Given its known involvement in the formation of ILVs, in yeast but also mammalian cells, our initial hypothesis was that the ESCRT family could be involved in the biogenesis of exosomes. In the meantime, however, evidences linking ESCRT components to the formation of ILVs have been challenged by some studies, which have put forth the notion that ESCRT-independent mechanisms exist. Regarding the Rab and SNARE families, their roles in intracellular trafficking of vesicles between different subcellular compartments, and between these compartments and the plasma membrane, suggested that they could play a role in the secretion process of exosomes. Indeed, when I started this project, the laboratory had just demonstrated the crucial role of 5 Rab proteins in exosome secretion, and especially the function of Rab27a and Rab27b in targeting and docking MVBs to the plasma membrane. But the molecular players of the final step of fusion of MVBs with the plasma membrane were not elucidated, and SNARE complexes represented good candidates to perform this task.

I have thus extended the original observations made in the laboratory on certain Rabs (Rab27a and Rab5 isoforms), which allowed me to evidence the heterogeneity of the vesicles released by cultured cells. In parallel, I made use of the screening technique that had been previously developed in our group, in order to analyze the effect of inactivating a large panel of ESCRT or of SNARE components in the model cell line HeLa-CIITA, on the amount of exosomes obtained in the cell conditioned medium. This allowed me to select a number of candidate proteins from the ESCRT family that seemed to be involved in the exosome pathway, and after validation characterize the secreted exosomes in more detail. Finally, I also used this screening to test the effect on exosome biogenesis or secretion, of other molecules described in the literature as potentially involved in this process.

The results obtained during my PhD studies will be presented in three parts:

Part 1: Presence of different subpopulations of exosomes in 100,000 x g pellets: Rab27a-dependent and -independent vesicles

Part 2: Function of ESCRT components in exosome biogenesis

Part 3: Function of other molecules, especially SNARE proteins, in exosome biogenesis or secretion

RESULTS

RESULTS

1) Presence of different subpopulations of vesicles in 100,000 x *g* pellets: Rab27a-dependent and -independent vesicles.

Summary

In the course of a study in which Rab27a invalidation was used to inhibit exosome secretion in murine tumor cells, we noticed the presence of different populations of secreted vesicles. We thus characterized in greater detail these vesicles by a combination of different techniques, including western blotting, immuno-electron microscopy and floatation on sucrose gradients. These techniques revealed the presence of at least two different populations of vesicles, which can be distinguished by their size, biochemical composition and secretion pathway: one population of smaller size (30-50 nm in diameter) was secreted independently of Rab27a function, and was enriched in the markers CD9 and Mfge8. The other population of vesicles showed a larger size (> 50 nm in diameter), the presence of several markers such as Alix, Tsg101, Hsc70 and CD63 in addition to CD9 and Mfge8, and its release required Rab27a activity.

Background

In addition to soluble molecules, cells can communicate between each other through the secretion of more complex structures, membrane vesicles. With sizes in the nanometer range, they consist of a membrane bilayer enclosing a small volume of cytosol. Different types of membrane vesicles have been described so far, and their intracellular origin is the main characteristic that allows us to differentiate exosomes, which are formed by inward budding of the limiting membrane of endosomes and are secreted upon fusion of these compartments with the plasma membrane, from other vesicles which are shed directly from the plasma membrane, such as microvesicles (Gyorgy et al., 2011). In an effort to understand the relevance of exosomes in physiological terms, great efforts have been made to better characterize these vesicles. This requires the purification of exosomes from cell conditioned media or biological fluids, and several protocols have been developed, including one that was used in the initial articles describing exosomes (Johnstone et al., 1987; Raposo

et al., 1996) and involves several centrifugation steps (Thery et al., 2006). After centrifugations at 300 x g and 2000 x g to eliminate unwanted cells and debris, the supernatant is centrifuged at 10,000 x g, a step that allows the retrieval of microvesicles, which are larger than exosomes (100-1000 nm in diameter vs. 50-100 nm for exosomes). Exosomes are pelleted at 100,000 x g; it is important to keep in mind that this achieves an enrichment in these vesicles, and in no way avoids the presence of similarly sized vesicles of a different origin, or of protein aggregates. The characterization of the obtained vesicles (Thery et al., 2006) involves the use of a combination of techniques aimed at the observation of the size and morphology (electron microscopy), and the enrichment in certain proteins that are considered as exosome markers (western blotting and immuno-EM). Floatation on an overlaid sucrose gradient allows further separation of vesicles with different densities.

The observation of exosomes by EM has shown that the size of these vesicles varies between 30 and 100 nm in diameter, in different cells types that have been analyzed: EBV-transformed B cells (Raposo et al., 1996), dendritic cells (Zitvogel et al., 1998), tumor cells (Wolfers et al., 2001), primary cortical neurons (Faure et al., 2006), etc. In addition to size heterogeneity, sucrose gradients (Escola et al., 1998) and immuno-EM staining of exosomes (Andre et al., 2004; Blanchard et al., 2002; Escola et al., 1998; Faure et al., 2006; Fevrier et al., 2004; Zitvogel et al., 1998) have also suggested the heterogeneity of these vesicles in terms of protein content.

We recently demonstrated that the small GTPases RAB27A and RAB27B are involved in exosome secretion in a human model (Ostrowski et al., 2010), HeLa-CIITA (HeLa cells expressing MHC class II molecules): treatment of these cells with shRNA specific for these proteins induced a significant decrease in exosome secretion, measured by the decrease of total protein content in exosome pellets, and the decrease of four exosomal markers (CD63, TSG101, MHC class II and HSC70). Here, a similar strategy was used to extend these findings to a murine model, and showed the presence of different vesicles in the 100,000 x g pellet.

Results and discussion

In order to inhibit exosome secretion by tumor cells, we employed shRNA-bearing lentiviruses to specifically invalidate Rab27a in the mouse mammary carcinoma 4T1. Of the shRNA tested to inhibit Rab27a expression, only one (sh2) induced a specific decrease of

Rab27a mRNA and protein as compared to cells expressing a control shRNA sequence (Scr), without affecting the expression of other proteins such as Rab27b (Article 1, Figure 1).

Exosome secretion was then measured: exosomes were purified from cell-conditioned medium of Rab27a-invalidated or control cells by the centrifugation protocol described earlier, and the 100,000 x g pellet obtained in the final step was analyzed by western blotting. Six markers were used to characterize the vesicles: in addition to markers regularly employed to describe exosomes secreted by human cells, such as CD63, Hsc70, Tsg101 and Alix, two markers described as highly enriched in mouse vesicles were employed, CD9 and Mfge8 (Article 1, Figure 2). Exosomes released by control cells showed the expected enrichment of Alix, CD63, CD9 and Mfge8, and the presence of Tsg101 and Hsc70. Upon Rab27a invalidation, four of the markers (Alix, Hsc70, CD63, and Tsg101) were significantly reduced compared to control cells, as expected from the results obtained in the human model. The other two markers, CD9 and Mfge8, were not significantly altered by the invalidation of Rab27a. Interestingly, these two proteins are not present in late endocytic compartments, as opposed to the other markers: Mfge8 is mostly a cytosolic protein and can bind the plasma membrane. The intracellular localization of CD9 was established by deconvolution microscopy (Article 1, Figure 6), which showed CD9 present in patches at the plasma membrane, as opposed to CD63 that was mainly accumulated in intracellular compartments.

The differences observed between the markers prompted us to ask if CD9 and Mfge8 could be present in different vesicles. We therefore allowed vesicles released by control and Rab27a knockdown cells to float into overlaid sucrose gradients, and analyzed the distribution of CD63, CD9 and Mfge8 among the different fractions that were obtained (Article 1, Figure 3). Western blotting analysis showed that Mfge8, CD9 and CD63 float at the expected sucrose density of exosomes (1.11 g/mL and mostly 1.14 g/mL). In the control conditions, Mfge8 is also abundant in the 1.09 g/mL fraction, whereas the tetraspanins are abundant in high density fractions (1.26-1.29 g/mL). CD63 was more enriched in the high density fractions than in the 1.11-1.14 g/mL fractions, the opposite was true for CD9. When we performed the same analysis with 100,000 x g pellets obtained from 4T1 cells invalidated for Rab27a, CD63 levels were now too low to be detected, and CD9 was more abundant in the 1.11 g/mL than in the 1.14 g/mL fraction. The 1.11 g/mL fraction was now more abundant in CD9 than the 1.14 g/mL fraction, and was also enriched in Mfge8; as

opposed to vesicles obtained from control cells, in which both proteins were more concentrated in the 1.14 g/mL fraction. These observations suggested that the vesicles present in the 1.14 g/mL fraction were dependent on Rab27a for their secretion, thus strengthening the hypothesis that different vesicles were present in the 100,000 x *g* pellet.

Finally, we observed the 100,000 x *g* pellet by immuno-EM. Antibodies against mouse CD63 and Mfge8 did not perform well in these conditions; therefore only the CD9 staining could be analyzed (Article 1, Figure 4). CD9 labeling was observed in vesicles of all sizes (30-150 nm in diameter), but was proportionally more abundant on vesicles smaller than 100 nm. This analysis also showed that in the 100,000 x g pellet obtained from Rab27a-invalidated cells, the amount of vesicles larger than 50 nm in diameter was decreased as compared to control vesicles, whereas vesicles smaller than 50 nm were equally abundant in both conditions. Thus, Rab27a is involved in the secretion of vesicles larger than 50 nm, and not of the smaller ones, which are enriched in CD9. Since Rab27a is involved in the exocytic fusion of MVEs with the plasma membrane (Ostrowski et al., 2010), it is possible that these smaller vesicles are derived from other intracellular compartments, or directly from the plasma membrane.

The analysis of the 10,000 x g pellet (Article 1, Figure 5), which contains vesicles usually described as microvesicles, showed that these particles contain CD9 and Mfge8 (CD63 was hardly detetctable). Rab27a inhibition did not alter the secretion of these two markers, as observed for exosome secretion (see Article 1, Figure 2). Staining of this pellet by immuno-EM indicated the presence of CD9 on vesicles with a size consistent with exosomes (under 100 nm diameter), as well as on larger vesicles.

Conclusions

This study demonstrated that different populations of vesicles are co-purified by the differential ultracentrifugation protocol used to purify exosomes by most groups. One population was dependent on Rab27a for its secretion, and possessed characteristics similar to those of exosomes: 50-100 nm diameter, a density around 1.14 g/mL, and an enrichment in CD63, CD9, Alix, Tsg101, Hsc70 and Mfge8. The other population was Rab27a-independent, had a smaller size (30-50 nm diameter), lighter density (around 1.11 g/mL), and was enriched in CD9 and Mfge8. The latter vesicles could have a different intracellular origin, and would therefore not be considered as exosomes.

Moreover, the analysis of microvesicles showed that they too are enriched in CD9 and Mfge8. It would thus appear that these proteins are not specifically enriched in exosomes, and should therefore not be considered as exosome markers.

These results are described in more detail in the article entitled "Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation" (Article 2), published in the Journal of Extracellular Vesicles.

Article 2 - Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation

Diverse subpopulations of vesicles secreted by different intracellular mechanisms are present in exosome preparations obtained by differential ultracentrifugation

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Exosomes are extracellular vesicles of 50 to 100 nm in diameter, released by many cell types. Exosomes are formed inside the cell in intracellular endosomal compartments and are secreted upon fusion of these compartments with the plasma membrane. Cells also secrete other types of membrane vesicles, for instance, by outward budding from the plasma membrane, and although some of them clearly differ from exosomes by their structural features (larger size), others are possibly more difficult to separate. Here, using Rab27a inhibition to modulate exosome secretion, we show the existence of at least 2 distinct populations of vesicles after purification by classical ultracentrifugation from mouse tumor cell conditioned medium. Rab27a inhibition lead to decreased vesicular secretion of some conventional markers of exosomes (CD63, Tsg101, Alix and Hsc70) but did not affect secretion of others (CD9 and Mfge8). By electron microscopy, CD9 was observed on vesicles of various sizes, ranging from 30 nm to more than 150 nm in diameter. Flotation onto sucrose gradients showed different proportions of CD63, CD9 and Mfge8 not only in fractions of densities classically described for exosomes (around 1.15 g/ml) but also in fractions of densities over 1.20 g/ml, indicating the presence of heterogenous vesicle populations. CD9 and Mfge8 were also found in large vesicles pelleted at low speed and can thus not be considered as specific components of endosome-derived vesicles. We propose that the most commonly used protocols for exosome preparations co-purify vesicles from endosomal and other origins, possibly the plasma membrane. Future work will be required to improve techniques for accurate purification and characterization of the different populations of extracellular vesicles.

Keywords: *extracellular vesicles*; *exosomes*; *Rab proteins*; *markers*; *secretion machinery*

To access the supplementary material to this article: Figures S1-S5, please see Supplementary files under Article Tools online.

In pluricellular organisms, cells constantly exchange informations to maintain integrity of tissues and accomodate changes in their environment. Secretion of soluble molecules, which bind to receptors on the surface of other cells, has been studied since the beginning of last century. But in the last decade, communication between cells via secretion of more complex structures such as membrane vesicles has become the focus of increasing interest from the scientific community (1–3). Membrane vesicles are composed of a lipid bilayer containing transmembrane proteins and enclosing soluble proteins and RNA, and the consequences of their

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interaction with cells are thus much more complex than the consequences of interaction of a single ligand with a single receptor. Extracellular membrane vesicles, called "microparticles" (4), have long been known in the blood circulation, where they were considered as cell fragments or pieces of membrane shed from the cell surface (2). But another type of vesicle secretion was described in the 1980s (5,6): it involved formation of vesicles inside endosomes, followed by extracellular release of these vesicles upon fusion of the endosomes with the plasma membrane. The resulting vesicles are of similar size as the internal vesicles of endosomes, i.e. between 50 and 100 nm in diameter, and are classically purified by ultracentrifugation at 70,000 or 100,000 g (7–9). The term "exosomes" has been used since 1987 to designate these extracellular vesicles of endocytic origin (8). A similar mechanism was also proposed to result in release of vesicles called "prostasomes" in semen (10).

Our groups have started working on exosomes secreted by immune cells, in the late 1990s (9,11,12), and have shown their potential as vehicles for antigen information within the immune system. We have also initiated the first proteomic analyses of exosomes secreted by dendritic cells (12) and shown the selective presence of a subset of intracellular proteins in these vesicles. A few years ago, we have published a detailed protocol for exosome purification by differential ultracentrifugation and characterization of the resulting vesicles by a combination of approaches showing enrichment of exosomal markers (electron microscopy and Western blotting) (13): this protocol was based on the original description of methods for isolating reticulocyte exosomes by the groups of Stahl (7) and Johnstone (8), and it is often referred to in publications dealing with exosomes.

In the last 4 years, we set out to identify the molecular machinery specifically involved in exosome secretion, in order to find ways to modulate this secretion and determine the physiological functions of exosome secretion in vivo. We have thus recently demonstrated a role of the small GTPases RAB27A and RAB27B in exosome secretion by a human tumor cell line (14). In this work, secretion of exosomes was measured by the release of a set of exosomal proteins, as defined by previous work from us (9,12) and others (15): major histocompatibility complex (MHC) class II molecules, the tetraspanins CD63 and CD81, heat shock protein cognate 70 (HSC70) and the protein involved in formation of multivesicular endosomes: tumor susceptibility gene 101 (TSG101).

Here, we used the same approach of Rab27a inhibition, in order to inhibit secretion of exosomes by mouse tumor cells. In addition to the proteins analyzed in human exosomes (14), we routinely characterize mouse exosomes using CD9 and milk fat globule – epidermal growth factor – factor 8 (Mfge8), 2 markers previously identified as

especially enriched in dendritic cell exosomes (12). Interestingly, this analysis allowed us to unravel a differential responsiveness to Rab27a inhibition of 3 markers, which are equally considered as specific of exosomes: 2 tetraspanins (CD63 and CD9) and a peripheral membrane-associated protein (Mfge8). Our work thus highlights the heterogeneity of the vesicle preparations commonly referred to as exosomes and shows that different structural and biochemical features are due to different molecular mechanisms of formation, and secretion.

Results

Selection of a shRNA sequence targeting specifically mouse Rab27a

We have shown recently that inhibition of RAB27A expression by stable transfection with shRNA in a human tumor cell line, HeLa-CIITA (a modified HeLa cell line expressing the MHC class II machinery), induces around 70% decrease in exosome secretion. This result was obtained by measuring the total amount of proteins recovered after 100,000 g ultracentrifugation, and the intensity of secretion of 4 classical markers of exosomes (CD63, TSG101, MHC class II and HSC70) (14). Here, to further extend these findings and to understand the role of exosome secretion by tumors in vivo (Bobrie, Théry et al., submitted mansucript), we analyzed the effect of Rab27a invalidation on exosome secretion by a mouse mammary carcinoma called 4T1. Following the same experimental design as in HeLa cells (14), we used lentiviruses expressing a puromycin-resistance gene and a shRNA sequence specific for *Rab27a*, or a control shRNA sequence, which does not target any mouse gene (Scr), to infect 4T1 cells. Out of 5 shRNA designed at inhibiting *Rab27a*, only 2 significantly inhibited this gene without affecting *Rab27b*, the other Rab27 isoform, in another mouse mammary carcinoma (Bobrie, Théry et al., submitted mansucript). We used these 2 shRNA in 4T1 cells and observed that one of them increased *Rab27b* expression (sh27a4, Fig. 1A). We thus used the only fully specific *Rab27a* shRNA sequence for the work described here and showed that it induced around 70% decrease of Rab27a protein in 4T1 cells (Fig. 1B).

Inhibition of Rab27a decreases secretion of a subset of exosome markers

Exosomes were purified by differential ultracentrifugation from the conditioned culture medium of Scr- or shRab27a-expressing 4T1 cells as previously described (13). The resulting 100,000 g pellet was analysed by Western blotting, using 6 different exosome markers (Fig. 2A). As expected, exosomes secreted by 4T1 cells are characterized by very strong enrichment of 2 tetraspanins (CD63 and CD9), Alix and Mfge8, as compared to cell lysates, and weaker (but clear) enrichment of
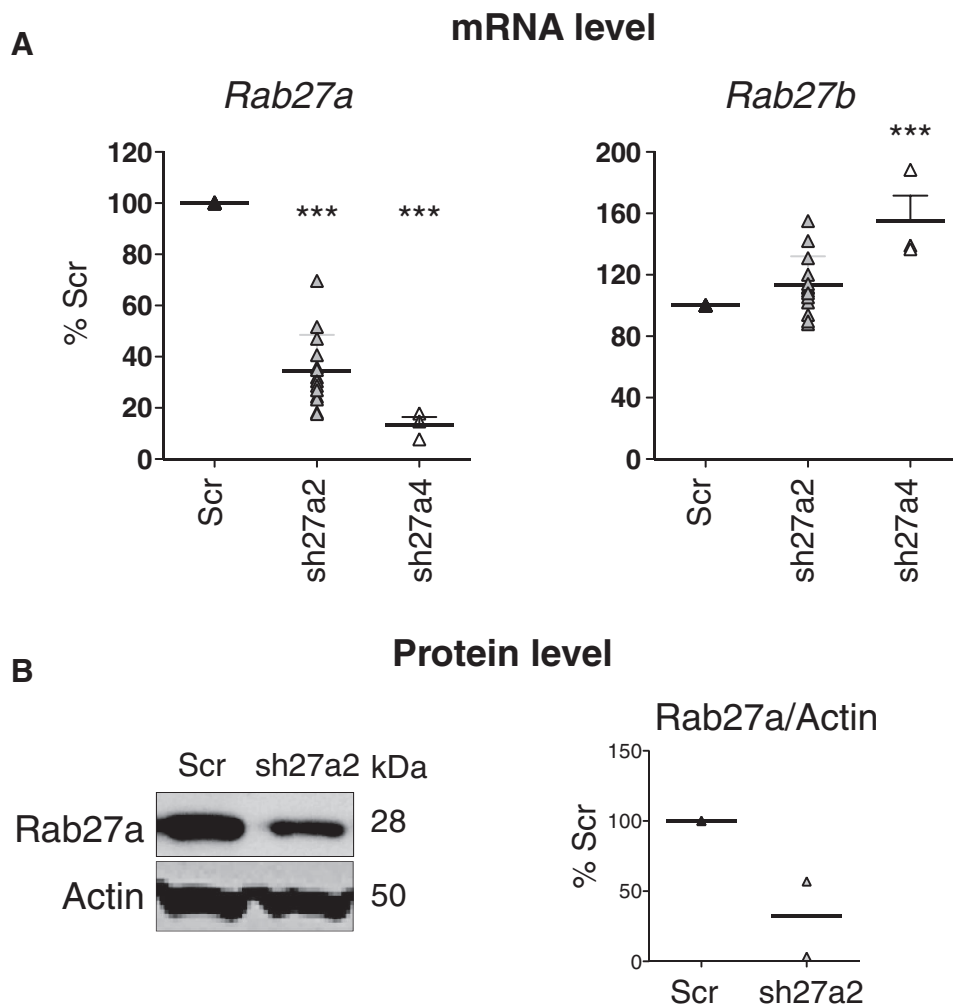
Fig. 1. Selection of a shRNA for specific inhibition of mouse Rab27a. A, Effect of 2 different shRNA sequences specific for *Rab27a* (sh27a2 and sh27a4) on *Rab27a* and *Rab27b* expression, measured by qRT-PCR. In each experiment, *Rab27* expression in shRab27-expressing cells is compared to expression in cells expressing a control shRNA (Scr). Individual results of 3 (sh27a4) or 7 (sh27a2) experiments are shown. Both sh27a2 and sh27a4 significantly decrease *Rab27a* expression, but sh27a4 also increases *Rab27b* expression. ***p < 0.01, 1-way ANOVA, Dunnett's post-test. B, Stable expression of sh27a2 induces downregulation of Rab27a at the protein level, as shown by Western blotting on total cell lysate (left panel). Actin is shown as loading control. Individual values of arbitrary units of Rab27a/actin band intensity in 2 independent experiments is shown (right panel). In each experiment, arbitrary units obtained for Scr cells are considered as 100%.

2 other classical exosome markers, Hsc70 and Tsg101. The endoplasmic reticulum-resident gp96 is used as a control for absence of cell debris in the exosome preparation. Significant decrease of secretion of 4 of these markers (CD63, Tsg101, Alix and Hsc70) was observed in shRab27a-expressing 4T1 (Fig. 2B). Surprisingly, secretion in the 100,000 g pellet of CD9 and Mfge8, 2 other markers classically used to characterize mouse dendritic or tumor cell exosomes (12,16), was not reduced in shRab27a-expressing cells (Figs. 2A and 2B).

CD63, CD9 and Mfge8 do not behave identically after flotation of the 100,000 g pellet on sucrose gradients
Since CD9 and Mfge8 did not behave like other exosomal markers, we asked whether they could be present on

different types of vesicles present in the 100,000 g pellet. A classical way to improve exosome purification after differential ultracentrifugation is to allow the pellet to float into an overlaid sucrose gradient (9,12,15,17). In these conditions, the original studies quoted above have shown that exosomal markers float at densities comprised between 1.11 and 1.19 g/ml, with slight variations of the position of the major fraction between different cell types and/or different markers. Here, we analysed the distribution of Mfge8, CD9 and CD63 among the sucrose gradient fractions, and we observed that the 3 markers did not behave identically. As shown in Fig. 3A, CD9 was present in all fractions from 1.09 to 1.29 g/ml, with a clear enrichment in the 1.11 and 1.14 g/ml fractions on one

A

	Scr		sh27a	
	Cell	Exo	Cell	Exo

Alix — 97 kDa
Hsc70 — 64 kDa
CD63 — 64 kDa / 51 kDa
Tsg101 — 51 kDa
Mfge8 — 51 kDa
CD9 — 28 kDa
gp96 — 97 kDa

B Alix ** Hsc70 ** CD63 * Tsg101
Mfge8 CD9
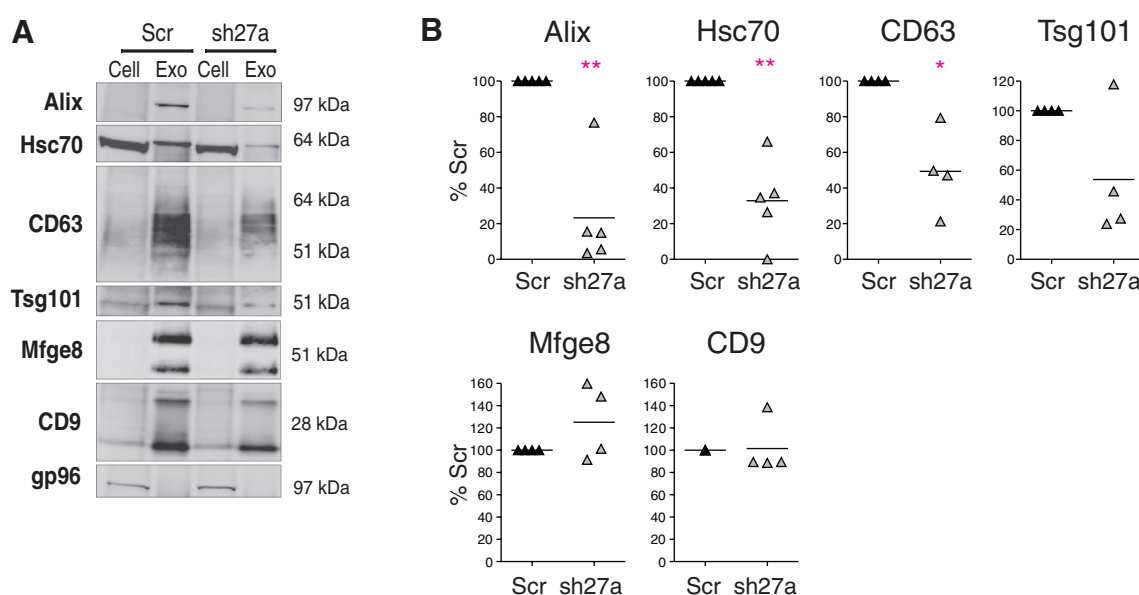
Fig. 2. Inhibition of Rab27a decreases secretion of a subset of exosomal markers. A, Western blot characterization of exosomes (Exo) secreted by the same number of control (Scr) or shRab27a-expressing 4T1 cells (sh27a), i.e. corresponding to 2–3 µg of total proteins. 30 µg of total cell lysates (cell) were analysed in parallel. Six different exosomal markers (CD63, Alix, Tsg101, Hsc70, CD9 and Mfge8) and a negative control (gp96) are shown. B, Quantification of the amount of each marker in exosomes obtained from shRab27a-expressing cells, as compared to control cells (individual results from 5 independent experiments are shown). *p < 0.05, **p < 0.01, paired t-test.

hand and, to a lower extent, in denser fractions of 1.26 and 1.29 g/ml. CD63 was also found in the same 2 sets of fractions, but it was much more abundant in the high density fractions than in the 1.11–1.14 g/ml fractions. Finally, Mfge8 was present throughout the sucrose gradient, with clear enrichment in the lightest fractions of 1.09, 1.11 and 1.14 g/ml, and no enrichment in the high density ones. When vesicles produced by shRab27a-4T1 were floated in parallel gradients (Fig. 3B), the level of CD63 was now too low to be detected, as expected from the decreased secretion level shown in Fig. 2, whereas CD9 and Mfge8 remained detectable. Interestingly, the 1.11 g/ml fraction now contained the highest amount of CD9 and as much Mfge8 as the 1.14 g/ml fraction, whereas, in vesicles from Scr-4T1, both proteins were most concentrated in the 1.14 g/ml fraction. These observations thus suggest that vesicles floating at 1.14 g/ml require Rab27a for their secretion, whereas the other fractions are not dependent on this machinery.

In addition, our results show that vesicles floating at different densities contain different relative amounts of the 3 markers. In particular, they are equally represented in the 1.14 g/ml fraction, but Mfge8 is more abundant than the 2 tetraspanins in the lowest density fraction (1.09 g/ml), whereas the high density fractions (above 1.26 g/ml) are very rich in CD63 and contain high amounts of CD9 and low amounts of Mfge8. Although such high density is normally considered to be specific of non-membranous materials like protein aggregates (which do not float into

sucrose), the interpretation is different for CD9 and CD63, which are tetraspanins containing 4 hydrophobic transmembrane sequences and thereby associated to membranes. A recent article by the group of Stoorvogel has shown that, in prostasomes, the CD9-containing material found at high sucrose density after a 16-hour centrifugation (as performed here) indeed corresponds to membrane vesicles, which end up floating around 1.15 g/ml if centrifugation is performed for 62 hours (18). Our observation that CD63 is also highly enriched in these fractions thus suggest that tetraspanin-enriched vesicles display a delayed flotation capacity, as compared to vesicles containing both tetraspanins and Mfge8.

CD9 is present on vesicles with different morphologies within the 100,000 g pellet

We then used immuno-electron microscopy (immuno-EM) to determine whether morphologically different vesicle populations are present in the 100,000 g pellets (Fig. 4). Although it works well on Western Blots, the antibody directed against mouse CD63 did not perform well in immunocytochemical analysis: it labelled less than 10% of the vesicles (Supplementary Fig. S5), whereas the anti-human CD63 used in our previous study (14) labelled around 80% of human vesicles (11). The anti-Mfge8 antibody showed an even weaker staining, with some background. We could thus not conclusively determine whether all or a subtype of vesicles bear CD63 or Mfge8. By contrast, labelling with the anti-CD9 antibody allowed us to investigate the distribution
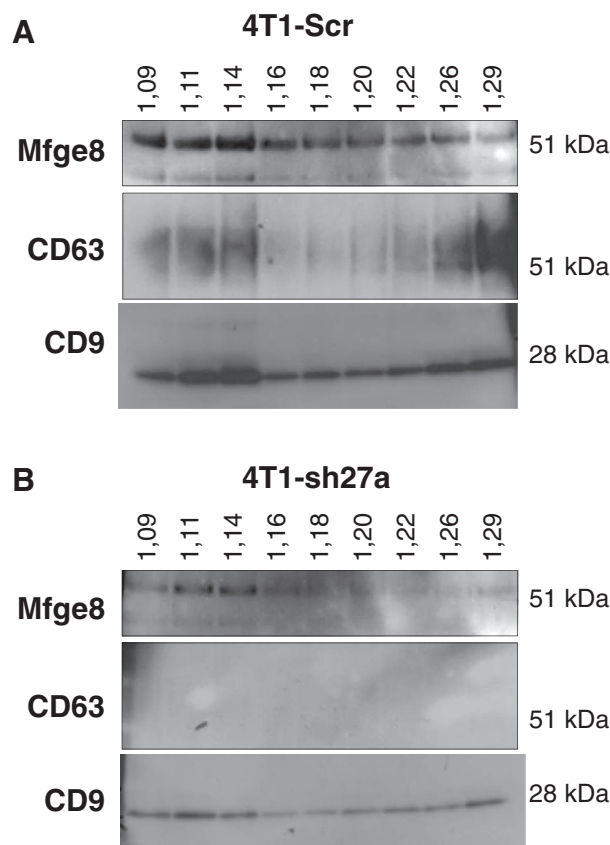
Fig. 3. CD9, CD63 and Mfge8 display subtly different patterns after floatation on sucrose gradients. A, The 100,000 g pellet recovered from Scr-4T1 cell conditioned medium was allowed to float into a sucrose gradient. The resulting fractions were analysed by Western blot using antibodies to Mfge8, CD63 and CD9. Density of each sucrose fraction in g/ml, measured by refractometry, is indicated above the gel. Mfge8, CD9 and CD63 float at the expected sucrose density of exosomes (1.11 g/ml and mostly 1.14 g/ml). Mfge8 also floats at 1.09 g/ml, whereas a large part of the tetraspanins also floats in high density fractions (1.26–1.29 g/ml). B, Same analysis performed with 100,000 g pellets obtained from shRab27a-expressing 4T1 cells. CD9 is more abundant in the 1.11 g/ml than in the 1.14 g/ml fraction and is also abundant in the high density fractions.

of this tetraspanin among the heterogenous population of vesicles. We observed gold particles labelling CD9 not only on 50–100 nm vesicles displaying a cup-shaped morphology as previously observed for exosomes (arrows) but also on smaller non cup-shaped (30–50 nm) (stars) and on some larger cup-shaped vesicles (over 150 nm) (Fig. 4A). The size of all vesicles in 5 random fields of EM pictures (Fig. 4B) and the number of CD9 particles on 200 isolated vesicles (Fig. 4C) were then analyzed. Clustered vesicles (see several examples in Fig. 4A) were excluded from this latter analysis because it is not possible to determine to which vesicle a given CD9 particle is bound when 2 vesicles are apposed. This analysis confirmed that vesicles of all sizes contain CD9 (Fig. 4C). It also showed that the number of CD9 particles per vesicle is not higher in the largest vesicles than in the 50–100 nm vesicles (average 2 versus 2.1 gold particles /vesicle in Fig. 4D), suggesting that CD9 may be proportionally more abundant on vesicles smaller than 100 nm, than on the bigger ones. Finally, this quantifica-

tion evidenced a decrease in the number of both 51–100 nm and larger vesicles in the 100,000 g pellet obtained from shRab27a-4T1 cells as compared to Scr-4T1, whereas vesicles smaller than 50 nm were as abundant in the 2 preparations (Fig. 4B). Thus, Rab27a is required for secretion of vesicles larger than 50 nm in the 100,000 g pellet and not for secretion of the smallest vesicles, which are enriched in CD9.

Rab27a inhibition does not affect secretion of large vesicles pelleting at 10,000 g

Since Rab27a is involved in the secretion of a subpopulation of the vesicles recovered in the 100,000 g pellet, we asked whether it was, or not, required for secretion of large membrane vesicles pelleted at 10,000 g [usually called "microvesicles" (1,17)]. Among the markers used to characterize exosomes, we routinely detected in the vesicles pelleting at 10,000 g only CD9 and Mfge8 (Fig. 5A). CD63 was sometimes also present, but at very low level (Fig. 5A), whereas Hsc70, Tsg101 and Alix were

A Exosomes from Scr 4T1 cells

CD9 staining

B

C 4T1-Scr 4T1-sh27a

D
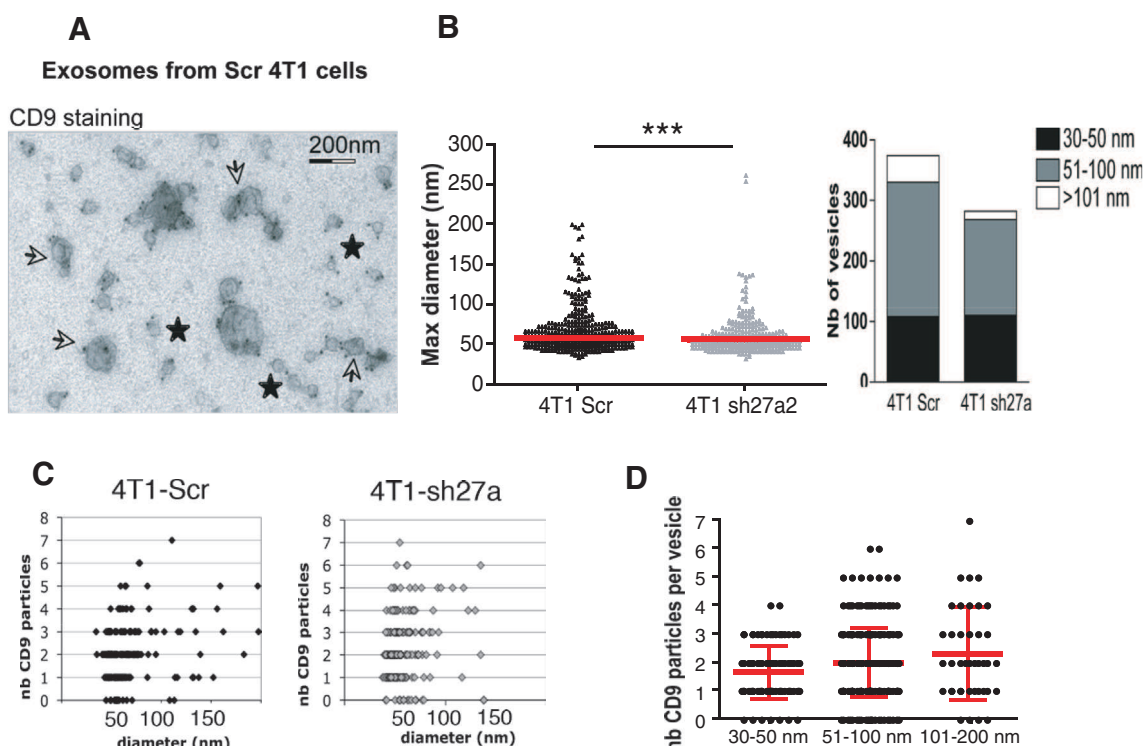
Fig. 4. CD9 is expressed on different types of vesicles present in the 100,000 g pellet. A–C, one representative Immuno-EM analysis of exosomes secreted by control (Scr) or sh27a-4T1 cells, labelled with anti-CD9 (10 nm). A, Representative image of exosomes from 4T1-Scr. Two populations of vesicles can be observed, displaying (arrows) or not (stars) the typical cup-shaped morphology. Diameter of the latter vesicles is below 50 nm. All vesicles are positive for CD9. Scale bar: 200 nm. B, The size of each individual vesicle was measured on EM pictures. The mean size of vesicles secreted by 4T1-sh27a is decreased (left panel, ***p < 0.001), due to a decrease in the proportion of vesicles larger than 50 nm (right panel). C, Quantification of the size and number of CD9 particles on isolated vesicles obtained from Scr-4T1 (left) or sh27a-4T1 cells (right). D, Quantification of the number of CD9 particles per individual vesicles, classified according to their size (30–50 nm, 51–100 nm, 101–200 nm). Results pooled from 2 individual experiments performed on vesicles from 4T1-Scr are shown. Mean + SD is displayed.

not detectable. Even though they were both present, the relative abundance of CD9 and Mfge8 was different in exosomes than in microvesicles, with lower amounts of CD9, as compared to Mfge8, in the 10,000 g than in the 100,000 g pellet (compare Exo and MV from the same preparation in Fig. 5A). As observed for exosomes (Fig. 2B), Rab27a inhibition did not affect the level of secretion of CD9 and Mfge8 in the microvesicles (Fig. 5B), whereas it decreased the low signal observed for CD63 (Fig. 5A), suggesting that CD63 is associated with the few exosomes possibly aggregated with larger vesicles during the 10,000 g centrifugation.

We then analyzed localization of CD9 by immuno-EM on vesicles pelleted at 10,000 g. Figure 5C shows that CD9 was present on vesicles of large size (above 200 nm), as well as on some vesicles with a size consistent with that of exosomes (below 100 nm) present in the 10,000 g pellet.

Our observations show that Rab27a is required for secretion of vesicles bearing endosomal markers (CD63, Alix and Tsg101), proteins also described in exosomes secreted by B lymphocytes (15), dendritic cells (11) and platelets (17). Rab27a, however, is not required for

secretion of smaller vesicles co-purified after differential 100,000 g ultracentrifugation, nor of larger vesicles pelleting at 10,000 g, which bear CD9 and Mfge8. In 4T1, CD63 is mainly present in intracellular compartments, whereas CD9 is found in patches at the cell membrane (Fig. 6): we thus propose that part of the CD9-bearing extracellular vesicles originate from the plasma membrane rather than from intracellular compartments. Our results also show that CD9 and Mfge8, even though they are abundant on the Rab27a-dependent exosomes, cannot be considered as specific exosome markers, since they are also present on other types of extracellular vesicles.

Discussion

The important conclusion from our work is the direct demonstration that different types of vesicles, the secretion of which is differently dependent on Rab27a, are co-purified by the differential ultracentrifugation protocol classically used by us and others to purify exosomes (13).

Previous EM analyses of exosome preparations revealed size heterogeneity (from 30 to 100 nm) in vesicles
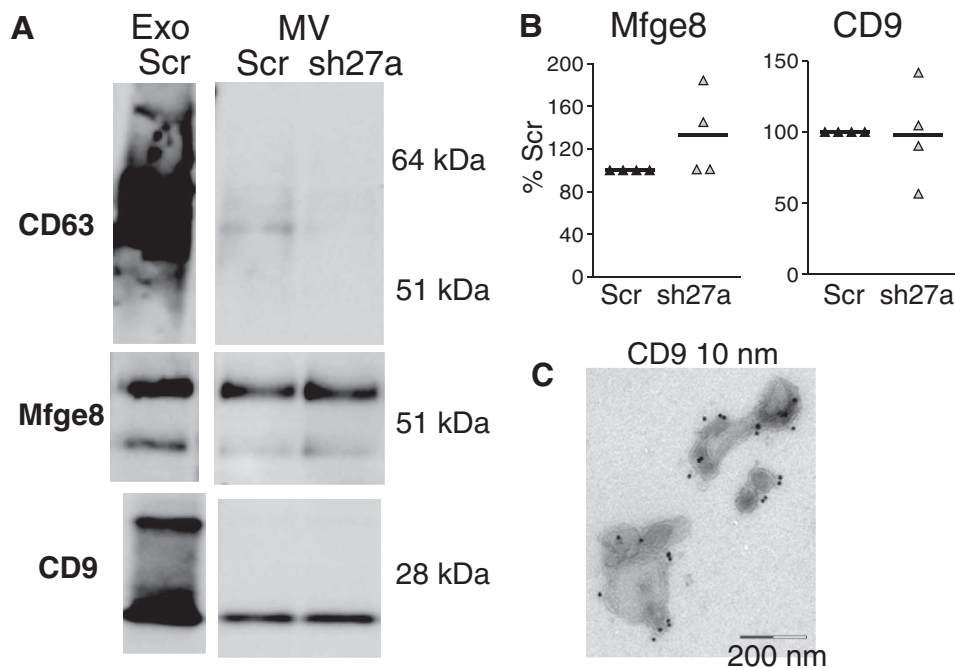
Fig. 5. Inhibition of Rab27a does not affect secretion of CD9 and Mfge8 in vesicles pelleted at 10,000 g. A, Western blot characterization of larger vesicles pelleted at 10,000 g (MV) secreted by 16×10^6 control (Scr) or sh27a-expressing 4T1 cells, as compared to the 100,000 g pellet (Exo) secreted by 15×10^6 Scr cells in the same experiment. One representative Western blot showing the presence of CD9 and Mfge8 in MV, whereas CD63 is hardly detected. B, Quantification of CD9 and Mfge8 in the 10,000 g pellet obtained from sh27a-expressing cells, as compared to control (Scr) cells (individual results from 4 independent experiments). C, Immuno-EM analysis of the 10,000 g pellet after staining with anti-CD9. CD9 is present on vesicles larger than 200 nm as well as on a few exosome-sized vesicles.

obtained by differential ultracentrifugation of supernatants from EBV-transformed B lymphocytes (9,15), platelets (17), mouse dendritic cells (11,12) or mastocytoma and melanoma tumor cell lines (19) among others. Thus, the size of exosomes was proposed to range from 30 to 100 nm, a variability that can be explained by the fact that multivesicular endosomes in some cell types contain intraluminal vesicles of heterogenous sizes. Size of the extracellular vesicles can thus not be used as a strict feature to define exosomes. In the 4T1 cells used here, we observed that secretion of the 30–50-nm vesicles is not affected by Rab27a inhibition. Since Rab27a is known to

be involved in regulated secretion from different types of intracellular compartments (20), we propose that, in the cells used here, the vesicles smaller than 50 nm do not come from intracellular compartments regulated by Rab27a. The small vesicles could thus either come from other intracellular compartments (in which case they can still be called exosomes) or directly from the plasma membrane (and we would not consider them as exosomes).

Since exosomes are smaller than the threshold of visualisation by confocal microscopy or by flow cytometry (about 200 nm), they could not be analysed reliably as single vesicles by any other methods than EM,
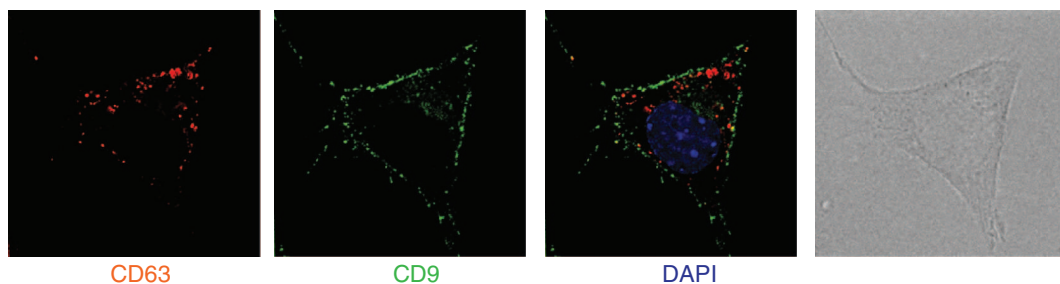
Fig. 6. CD9 and CD63 are not present in the same intracellular locations. 4T1 cells were analysed by deconvolution microscopy after staining with anti-CD9 (green) or anti-CD63 (red). Position of the nucleus is shown by DAPI staining (blue). CD9 is mainly expressed in small patches at the cell surface, whereas CD63 accumulates in intracellular compartments.

which limited the possibilities to determine whether the differently sized vesicles were also different in terms of protein markers. Thus, it was accepted so far to characterize bulk populations of vesicles by the presence, or not, of a set of exosomal protein markers, which, if enriched in the vesicle preparations as compared to the cells secreting them, qualified vesicles as exosomes (13). But, we now show here that 2 of these exosome markers, CD9 and Mfge8, are not specific of vesicles derived from intracellular multivesicular compartments, since they are also present on larger vesicles that pellet at 10,000 g in the sequential ultracentrifugation protocol, and their secretion is not significantly affected by Rab27a inhibition as opposed to secretion of other proteins, such as CD63, Hsc70, Tsg101 and Alix (see Fig. 2).

In most cells, the bulk of CD63 is present in intracellular compartments of endosomo/lysosomal origin (21), which is also the case in the 4T1 cells used in this study (Fig. 6). CD9, by contrast, is mainly observed in 4T1 cells at the cell surface or in vesicles near the cell surface, as shown in Fig. 6. In other cells, CD9 has been described both at the cell surface and intracellularly in multivesicular endosomes (22,23). In mammary epithelial cells (24), Mfge8 not only is present throughout the cytoplasm but also binds to spots of phosphatidylserine at the cell surface, and it was shown also to associate to vesicles of different sizes, both larger and smaller than 100 nm. Thus, we propose that small vesicles budding directly from the plasma membrane, as well as vesicles formed in intracellular compartments and released by their fusion with the plasma membrane, are simultaneously secreted by the cells: ultracentrifugation at 100,000 g pellets both types of vesicles without specificity.

CD9, Mfge8 and Hsc70 had been identified in exosomes from dendritic cells in the first proteomic analysis of these vesicles that we published in 1999 (12) and Tsg101 and Alix in our following article (25). Comparative analysis, by Western blotting, of the level of these proteins in the secreting cells and their exosomes had evidenced the strong enrichment of CD9 and Mfge8 (12,26) and the clear (but weaker) enrichment of Hsc70 (12), Alix (25) and Tsg101 (27) in the exosome preparations. These observations, and the consistent identification of all these markers in numerous subsequent proteomic analyses of exosomes [analyses compiled in the Exocarta website, described initially in (16)], had led to propose these proteins as markers of exosomes. In most analyses, however, separation of the vesicles on sucrose gradients had not been performed, and the preparations contained both Rab27a-dependent and -independent vesicles. In addition, in none of these studies were exosomes and larger vesicles compared side-by-side; hence, the specificity of a given protein as a marker of exosomes, as opposed to other extracellular vesicles, was not strictly addressed. In our own previous studies, for instance, we used a portion of Mfge8 [the C1C2 domain as described in (28)] to target an antigen to secreted extracellular vesicles, and we showed that exosomes bore high amounts of the antigen (29). But the results presented here suggest that other extracellular vesicles would probably, in vivo, also bear this antigen. Thus, the increased efficiency of immune responses induced by the modified antigen in vivo could not be specifically attributed to its secretion on exosomes but more generally on extracellular vesicles. Other groups have since used the C1C2-based modification of antigens (30,31), and in these studies too, the specificity of targeting to exosomes should be re-evaluated.

Therefore, new analyses of all the proteins commonly found in exosomes should now be performed, to determine their major flotation density on sucrose gradients as compared to CD9, CD63 and Mfge8, and their enrichment on larger vesicles excluded from the exosome preparations. Such proteins include, for instance, acetylcholinesterase (AChE) or placental alkaline phosphatase (PLAP), described previously on reticulocyte (8) or tumor exosomes (32,33), but which are also present in the cell membrane (8). Density in sucrose of other proteins identified in several proteomic analyses and used randomly as markers of exosomes by different laboratories should also be determined: for instance, annexins (e.g. annexin II), and lipid raft-associated flotillins. These analyses will constitute a first step into defining the required characteristics of specific exosomal markers. Establishment of more specific purification procedures will, however, be necessary to establish a strict definition of specific exosome markers.

Indeed, none of the currently used protocols of exosome purification are likely to separate the different types of vesicles we describe here. A technique involving size-exclusion chromatography before ultracentrifugation at 100,000 g (34) allows elimination of proteins but does not separate large from small vesicles, thus a fortiori will not differentiate small vesicles of different densities or compositions. Several groups, including ours (14,27,35), include a filtration step over 0.22 μm filters during exosome purification: although such filtration eliminates large vesicles (as well as aggregated small vesicles), it will not separate the different vesicles of a size smaller than 220 nm. Finally, a recently commercialised method called Exoquick has been developped, aiming at fast and easy purification of exosomes. But, since the vesicles recovered bear large amounts of CD9, but low amounts of CD63, as shown on Western blots (http://www.systembio.com/microrna-research/exoquick-exosomes/technical-details), we speculate that this procedure is not specific for endosome-derived exosomes.

The use of sucrose gradients allows some separation of the different vesicles. We have observed here that, as recently described by others (18), some vesicles with high content of tetraspanins (CD9 and CD63) but low level of Mfge8 display an apparent high density in sucrose (above 1.23 g/ml). Whether these vesicles come from internal vesicles of intracellular compartments, or from portions of the plasma membrane, maybe corresponding to lipid raft-enriched subdomains, remains to be determined.

But, in addition, we observed subtle differences in the distribution of 3 membrane-associated proteins within the classical range of densities described for exosomes, i.e. 1.11 to 1.19 g/ml. Examples of slightly different densities observed for different exosomal markers from the same cellular source have been published. When analyzing simultaneously MHC class II molecules and CD81 and CD82 tetraspanins in B-EBV exosomes, Escola et al. (15) showed that the 1.13 g/ml fraction was the most enriched in MHC class II molecules, whereas CD81 was equally distributed between the 1.13 and 1.14 g/ml fractions, and CD82 between the 1.12 and 1.13 g/ml fractions. In the same line, we show here that CD9- and CD63-containing vesicles float mainly at 1.14 g/ml and to a lower extent at 1.11 g/ml, whereas Mfge8 is present equally in the same 2 fractions and a lighter one of 1.09 g/ml. In addition, we show that the CD9-containing vesicles floating at 1.14 g/ml depend on Rab27a for their secretion, whereas the vesicles floating at 1.11 g/ml are less affected by Rab27a inhibition.

Based on our results and on re-analysis of previously published work, we want to propose that sucrose gradients are not resolutive enough to separate vesicles with small differences in densities, which use different intracellular machineries for their secretion. Since they have different protein compositions, we think that the different subpopulations of extracellular vesicles, in particular, the Rab27a-dependent and -independent vesicles, should display different physiological functions. Therefore, it will be crucial in the future to design novel methods for purification and characterization of the different types of vesicles, to allow precise analysis of their respective functions.

Materials and methods

Cells

4T1 cells were cultured in RPMI (Life Technologies) supplemented with 10% fetal calf serum (Abcys) 2 mM glutamine, penicillin/streptomycin (100 U/mL and 100 µg/mL respectively), and 1 mM pyruvate. For stable inhibition of Rab27a expression, cells infected with shRNA-expressing lentiviruses were selected and maintained in medium containing 5 µg/mL puromycin (Life Technologies). Cells were used within 1 month after lentivirus infection. Independent experiments were performed with batches of independently infected cells.

Reagents

Antibodies used for Western blotting: Hsc70 and gp96 (Stressgen Biotech), Tsg101 (Genetex, clone 4A10), anti-mouse CD63 (MBL, clone R5G2), Alix/AIP1 (Acris antibodies, clone 49/AIP1), anti-mouse CD9 (BD Pharmingen, clone KMC8), anti-mouse Mfge8/lactadherin (MBL, clone 18A2-G10) and actin (Millipore, clone C4). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Immunoresearch except the HRP-conjugated anti-hamster antibody, which was purchased from Santa Cruz Biotechnology. Mouse anti-Rab27a was kindly provided by C. Recchi and M. Seabra (20). The same anti-CD9 and CD63 were used for immunofluorescence.

pLKO.1puro plasmids allowing expression of shRNA specific for mouse Rab27a, or a scrambled sequence of shRNA to GFP as control (Scr), and a puromycin-resistance gene (36) were kindly provided by L. F. Moita. Lentiviruses were produced as previously described and used to infect subconfluent cells in 96-well plates. Sequence of the shRNA 27a2: CCGG**CGAAACTGGAT AAGCCAGCTA**CTCGAG**TAGCTGGCTTATCCAGTT TCG**TTTTTG Sequence of the shRNA 27a4: CCGG **CCAGTACACTGATGGCAAGTT**CTCGAG**AACTTGC CATCAGTGTACTGG**TTTTTG

Quantitative RT-PCR

Total RNA were purified from 10^6 cells using Macherey Nagel Nucleospin RNA II kit; 200 ng, as measured by Nanodrop (Thermo Scientific), was reverse transcribed with SuperScript II (Invitrogen); 1/20th of the obtained cDNA was used for quantitative PCR, performed in triplicate, in Absolute Q-PCR SYBR Green ROX Mix (Abgene) on a Lightcycler LC480 (Roche). Primers used were purchased from Qiagen (QuantiTect Primer Assay). Cycle threshold (Ct) for *Rab27a* were normalized to Ct for *Gapdh*, and results were expressed as percentage of the control shRNA-transduced cells.

Exosome purification and characterization

Exosome purification was performed as previously described (13). Briefly, cells plated in 145 mm² plates were incubated 48 hours in medium depleted from serum-derived exosomes and microvesicles by overnight ultracentrifugation at 100,000 g. Supernatants were collected and exosomes were purified by successive centrifugations: 300 g for 10 minutes; 1,000 g for 20 minutes; 10,000 g for 40 minutes and 100,000 g for 90 minutes. The 10,000 g and 100,000 g pellets were washed in large volumes of PBS and resuspended in 80 µl PBS. Producing cells were counted and proteins [obtained by lysing cells in 50 mM Tris, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, 0.1% sodium azide, with a cocktail of antiproteases (Roche)]

were prepared from 4×10^6 cells. Proteins in pellets and lysates were quantified by Micro-BCA (Thermo Scientific) in the presence of 2% SDS. Exosomes secreted by 10×10^6 to 17×10^6 cells (i.e around 3 μg), microvesicles (10,000 g pellet) obtained from 15×10^6 cells or 30 μg of lysates (corresponding to around 2.10^5 cells) were loaded on NuPAGE 4–12% BisTris gels and separated under non-reducing conditions. For Rab27a detection by Western blotting, 150 μg of lysates were analyzed in reducing conditions. Intensity of bands on Western blots was quantified using QuantityOne or ImageJ softwares.

Electron microscopy (EM)
Immuno-EM analysis was performed as previously described (13), on pellets of purified exosomes loaded on formwar/carbon-coated grids and fixed in 2% paraformaldehyde. Grids were labelled with rat anti-mouse CD9 (KMC8, 10 μg/ml), followed by rabbit anti-rat (Dako, 1/200) and protein A-10 nm gold (UMC Utrecht, 1:60). Grids were observed at 80 kV with a CMV120 Twin Philipps Electron Microscope (PEI Company). Size of individual vesicles was measured on 5 different pictures taken at $10,000 \times$ magnification for each exosome preparation, using the iTEM software.

Separation of vesicles on sucrose gradient
Exosomes purified from $\pm 10^8$ cells were resuspended in 2 ml PBS-2.5M sucrose, loaded in a SW41 tube and overlaid with 15 successive 750 μl layers of PBS containing decreasing concentrations of sucrose (from 2 to 0.4 M). Tubes were centrifuged for 16 hours at 4°C at 200,000 g. One millilitre fractions were collected; sucrose density was measured on an aliquot of each on a refractometer, and fractions were diluted in 3 ml PBS, ultracentrifuged at 100,000 g for 1 hour and resuspended in PBS before analysis by SDS-page and Western blotting.

Immunofluorescence microscopy
4T1 cells seeded on glass coverslips were fixed with 4% formaldehyde, before staining with the same anti-CD9 and anti-CD63 antibodies as for Western blots. Stainings were performed successively with anti-CD9 followed by anti-rat-Alexa488, postfixation in formaldehyde, and anti-CD63 followed by anti-rat-Alexa568. Washes were performed in PBS-0.1% BSA-0.05% Saponine and cells were stained with DAPI 1 μM before mounting. Images were acquired with a wide-field Eclipse 90i Upright Microscope (Nikon) equipped for image deconvolution. Acquisition was performed using a $100 \times$ Plan Apo VC 1.4 oil objective and a highly sensitive cooled interlined charge-coupled device (CCD) camera (Roper CoolSnap HQ2). Z-dimension positioning was accomplished by a piezoelectric motor (LVDT, Physik Instrument) and a z-dimension series of images was taken every 0.2 μm. Deconvolution was performed according to Ref. (37).

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2) Function of ESCRT components in exosome biogenesis

Summary

In order to assess the potential role of ESCRT proteins in exosome biogenesis, we performed an RNA interference screening, which targeted 23 individual ESCRT components. Secretion of CD63, MHC class II and/or CD81-bearing exosomes was reduced in cells invalidated for HRS, STAM1 and TSG101, and was increased in cells with CHMP4C, VPS4B, VTA1 and ALIX loss of function. A more detailed study of the exosomes secreted by cells invalidated for these ESCRT proteins showed, in addition to the presence of different populations of vesicles, different types of modifications of size and protein content of the secreted vesicles. Particularly, inactivation of ALIX induced an enrichment of MHC class II molecules in the secreted vesicles, accompanied by a general increase of this molecule in the vesicle-secreting cells.

Background

Exosomes were first described as vesicles originating in the endocytic pathway, during the maturation of early endosomes to form MVEs. The formation of ILVs, which when secreted by fusion of MVEs with the plasma membrane are termed exosomes, involves lateral segregation of cargoes at the limiting membrane and the formation of buds towards the lumen of the endosomes (Stoorvogel et al., 2002). Much effort has been made towards identifying the molecular machinery responsible for this process. The first machinery to be described was the ESCRT machinery, initially identified as responsible of the formation of MVEs in the yeast (Hanson and Cashikar, 2012). It comprises four proteins complexes (ESCRT-0, -I, -II and -III) and accessory proteins that interact with each other and are transiently recruited to the limiting membrane of endosomes. ESCRT-0, -I and -II have been linked to the clustering of ubiquitinated cargoes into microdomains, and ESCRT-III is thought to be involved in the inward budding and scission of vesicles (Wollert et al., 2009).

Several evidences of ESCRT function in the biogenesis of exosomes have been reported. The proteomic analysis of exosomes from different origins has shown the presence of ESCRT components (Tsg101, Alix), as well as ubiquitinated proteins. The ESCRT-0 component Hrs has been shown to regulate in DCs the secretion of exosomes induced by OVA or calcium ionophores (Tamai et al., 2010). Furthermore, ALIX has been shown to mediate the sorting

of the TfR into exosomes in reticulocytes (Geminard et al., 2004), and more recently, to be involved in the biogenesis of exosomes through its interaction with syntenin (Baietti et al., 2012).

However, several groups have observed the presence of different populations of endosomes within cells (Buschow et al., 2009; Mobius et al., 2003; White et al., 2006), suggesting that diverse mechanisms might participate in the formation of these different compartments and thus implying that pathways other than ESCRTs might be involved. In fact, evidences of different mechanisms have emerged in the past few years, including ceramide (Trajkovic et al., 2008) and tetraspanins (van Niel et al., 2011). Moreover, inactivation of the four ESCRT complexes did not completely impair MVE formation (Stuffers et al., 2009), further strengthening the idea that other pathways may be involved. MVE biogenesis and consequently exosome formation are therefore still poorly understood.

We addressed the involvement of ESCRT complexes in exosome biogenesis by using an RNA interference screen targeting individual ESCRT and accessory proteins, in HeLa-CIITA cells expressing MHC class II molecules.

Results and discussion

We performed a screen as previously set up in our group (Ostrowski et al., 2010), targeting 23 individual ESCRT proteins by shRNA treatment in HeLa-CIITA cells, and we monitored exosome secretion by quantifying MHC class II and/or CD81-positive vesicles trapped on anti-CD63-coated beads. Gene down-regulation was verified by qRT-PCR. Among the 23 genes that were analyzed (Article 2, Table 1), we failed to demonstrate a reliable down-regulation for 7 of these genes, which were thus not analyzed for their effects on exosome secretion. Four of the candidates did not induce a significant modulation of exosome secretion as compared to cells treated with the control shRNA SCR (Article 2, Figure S1: VPS28, VPS37A, SNF8/VPS22, VPS25), and five of them showed inconsistent effects of the different shRNA on exosome secretion (Article 2, figure S2: STAM2, VPS36, CHMP1B, CHMP5, CHMP6/VPS20). By contrast, seven candidates were shown to have an effect on exosome secretion, since at least 2 different shRNA sequences efficiently inhibited gene expression, and modulated exosome secretion in the same manner (Article 2, Figure 1). Invalidation of early components of the ESCRT machinery, HRS, STAM1 and TSG101, induced

a significant decrease in exosome secretion. Conversely, inhibition of late acting components, CHMP4C, VPS4B, VTA1 and ALIX, induced an increase in exosome secretion.

To validate these results, we chose one shRNA sequence of each candidate to invalidate gene expression (Article 2, Figure S3), and purified the vesicles secreted by large numbers of cells, using the differential centrifugation protocol described previously. Western blotting analysis of three exosome markers, MHC class II, CD63 and HSC70 (Article 2, Figure 2), showed that the secretion of all markers was decreased in HRS-, STAM1- and TSG101-invalidated cells as compared to the control, suggesting a decrease in exosome secretion by these cells. CHMP4C and VTA1 inhibition induced no change in exosome secretion, which is contrary to the initial conclusion of the screen. VPS4B inhibition displayed an increased secretion of all markers, whereas ALIX knockdown induced the increase of MHC class II but not of the other two markers, which pointed to a change in exosome composition rather than an effect on exosome secretion.

Immuno-EM was performed to compare the morphology and CD63 and MHC class II content of the vesicles released by control and shRNA-treated cells (Article 2, Figures 3 and S4). Interestingly, we observed that at least two different types of vesicles coexisted in the 100,000 x g pellet regardless of the condition: smaller vesicles (30-60 nm diameter) were enriched in CD63, as compared to larger vesicles > 100 nm diameter) that had a higher MHC class II content. Moreover, invalidation of the ESCRT components induced differences in the size of the secreted vesicles as compared to the control: HRS invalidation increased the proportion of smaller vesicles (30-60 nm), whereas invalidation of STAM1, TSG101, VPS4B, VTA1 and ALIX increased the proportion of large vesicles (> 100 nm) in the exosome preparations. When analyzing the size and protein content (CD63 and MHC class II) simultaneously, shRNA against VPS4B, HRS or STAM1 did not alter the proportion of vesicles bearing each marker, together or separately, as compared to the control. By contrast, TSG101 inactivation caused a decrease in the proportion of CD63- and MHC class II-bearing vesicles. Finally, the shRNA against ALIX induced an increase in MHC class II content in vesicles of all sizes, which is in agreement with the previous results.

The striking effects observed upon invalidation of ALIX warranted an analysis of the secreting cells (Figure 4). Western blotting performed on total cell lysates from control and shALIX-treated cells confirmed the decrease of ALIX and the increase of MHC class II in treated cells. A surface FACS staining showed the increase of MHC class II at the cell surface,

whereas CD63 levels remained stable, and immuno-EM performed on ultrathin cryosections evidenced the increase of MHC class II in MVEs as well. To find out if the increase in MHC class II observed in HeLa-CIITA cells could be reproduced in another cell type, we treated human primary monocyte-derived DCs with control and ALIX shRNA (Article 2, Figure 5). The analysis of these cells by western blotting and FACS analysis for cell surface expression showed that immature DCs sustain an increase in total and surface MHC class II upon ALIX invalidation, which cannot be further increased by maturation. The FACS-based assay previously used with the HeLa-CIITA model was employed to assess if ALIX invalidation of DCs also induced an increase in MHC class II secretion on CD63-bearing exosomes: the results obtained with cells from seven donors showed it was not the case, suggesting that the subpopulations of vesicles within DC and HeLa-derived exosomes may be different. The combined results observed in HeLa-CIITA and dendritic cells suggest that ALIX is involved in the trafficking of MHC class II molecules in different cell types, and might also impact on the sorting of proteins into the ILVs, at least in the HeLa-CIITA model.

Conclusions

The effects observed upon invalidation of the seven ESCRT proteins highlight the requirement for selected ESCRT components in the biogenesis of exosomes.

These results are described in more detail in the manuscript "ESCRTs differentially modulate exosome biogenesis, composition and secretion in MHC class II-expressing cells" (Article 3, in preparation).

Article 3 - ESCRTs differentially modulate exosome biogenesis, composition and secretion in MHC class II-expressing cells

ESCRTs differentially modulate exosome biogenesis, composition and secretion in MHC class II-expressing cells

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ABSTRACT

Exosomes are small membrane vesicles secreted upon fusion of multivesicular endosomes with the plasma membrane. They are involved in several processes from immunity to neurodegenerative disorders and start to be used in clinical trials for therapeutic purposes. The mechanisms involved in exosome biogenesis remain so far unclear even though they constitute potential targets to modulate exosome production and secretion. Here we have performed an RNA interference (RNAi) screen targeting components of the Endosomal Sorting Complex Required for Transport machinery (ESCRT-0/I/II/III) and associated proteins (VPS4, VTA1, ALIX) in HeLa-CTIIA cells expressing MHC class II molecules. The results of the screen indicated that secretion of exosomes, defined as MHC class II and/or CD81-positive vesicles captured by anti-CD63-coated beads, was decreased upon inactivation of HRS, STAM, TSG101, but increased upon CHMP4C, ALIX, VPS4B loss of function. As evaluated by western blot and electron microscopy, targeting of the different ESCRT components also consequently impacted on the size and protein content of the released vesicles. Inactivation of ALIX considerably modified the nature and composition of the secreted exosomes. Cells inactivated for ALIX released a higher proportion of large vesicles as compared to control cells, and the secreted vesicles were enriched in MHC class II, whereas smaller vesicles to which CD63 associates remained unchanged. We further showed that ALIX inactivation leads to an increase in the amount of MHC class II at the plasma membrane and intracellular compartments, which was also observed in primary dendritic cells. The results together reveal an involvement of selected ESCRT components in exosome secretion and highlight ALIX as a potential candidate to manipulate MHC class II trafficking in cells, resulting in a different molecular composition and possibly intracellular origin of the secreted vesicles.

INTRODUCTION

Exosomes correspond to the intralumenal vesicles (ILVs) of multivesicular endosomes (MVEs), thereby they are formed within the endocytic pathway during the maturation of endosomes. While some MVEs are fated for degradation, one population fuses with the plasma membrane in an exocytic manner, a process during which the ILVs are secreted as exosomes. The generation of the ILVs into MVEs involves the lateral segregation of the cargo at the limiting membrane of an endosome, by the formation of an inward budding vesicle and the release in the endosomal lumen of the membrane vesicle containing a small portion of cytosol. Over the past 10 years several studies have started to elucidate the mechanisms involved in ILV formation and cargo sorting (Hanson and Cashikar, 2012; Henne et al., 2011; Hurley, 2008; Raiborg and Stenmark, 2009). Given that exosomes correspond to ILVs, the same mechanisms are supposed to be involved in their biogenesis. However, cells host different populations of MVEs and ILVs (Buschow et al., 2009; Mobius et al., 2003; White et al., 2006) and we are still at an early stage of understanding exactly what are the mechanisms that contribute to exosome formation and cargo sorting within these vesicles.

The first machineries described to be involved in MVB and ILV biogenesis are the ESCRT (Endosomal Sorting Complexes Required for Transport) complexes. The ESCRT family comprises approximately 20 proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins conserved from yeast to mammals (Roxrud et al., 2010; Saksena et al., 2007). The ESCRT-0, -I and -II complexes recognize and sequester ubiquitinated proteins in the endosomal membrane, whereas the ESCRT-III complex seems to be responsible for the membrane budding and vesicle scission (Wollert et al., 2009). ESCRT-0 consists of Hrs that recognizes the mono-ubiquitinated cargo proteins and associates in a complex with STAM, Eps15 and clathrin**.** Hrs recruits Tsg101 of the ESCRT-I complex, and ESCRT-I is then involved in the recruitment of ESCRT-III, via ESCRT-II or Alix, an ESCRT-accessory protein. Finally, the dissociation and recycling of the ESCRT machinery requires interaction with the AAA-ATPase Vps4. It has been unclear whether ESCRT II has a direct role on biogenesis of ILVs or its sorting function could be just limited to a particular cargo (Bowers et al., 2006; Malerod et al., 2007).

Studies in oligodendrial cell lines secreting the proteolipid PLP in association with exosomes (Trajkovic et al., 2008) and in melanocytic cells producing melanosomes (Theos et al., 2006) highlighted that mammalian cells developed additional pathways of MVE/ILV formation. Moreover, concomitant inactivation of ESCRT subunits belonging to the 4 ESCRT complexes does not totally impair the formation of MVEs indicating that other mechanisms are thought to operate in the formation of ILVs and thereby of exosomes (Stuffers et al.,

2009). One of these pathways is thought to require a type II sphingomyelinase that hydrolyzes sphingomyelin to ceramide (Trajkovic et al., 2008). Whereas the depletion of different ESCRT components did not lead to a clear reduction in the formation of MVEs and in the secretion of PLP via exosomes, inhibition of sphingomyelinase expression with siRNA or its activity with the drug GW4869 decreased exosome formation and release. However, whether such dependence on ceramide is generalizable to other cell types producing exosomes has not been further strengthened. Inactivation of type II sphingomyelinase in melanoma cells does not impair MVB biogenesis (van Niel et al., 2011) and exosome secretion (our unpublished data).

Proteomic analyses of purified exosomes, from various cell types, show the presence of ESCRT components (Tsg101, Alix) and ubiquitinated proteins (Buschow et al., 2005; Thery et al., 2001). Also, it has been reported that the ESCRT-0 component Hrs, could be required for exosome formation/secretion by dendritic cells and thereby impact on their antigen presenting capacity (Tamai et al., 2010). The Transferrin Receptor in reticulocytes that is generally fated for exosome secretion, although not ubiquitinated, interacts with Alix for MVE sorting (Geminard et al., 2004). More recently it was also shown that Alix is involved in exosome biogenesis and exosomal sorting of syndecans via its interaction with syntenin (Baietti et al., 2012).

However, as discussed above and elsewhere (Bobrie et al., 2011) and mostly because in most studies only one or few selected ESCRT subunits and accessory proteins have been analyzed, it is still unclear whether ESCRT components could be involved in the formation of exosomes. We have here carried a medium-throughput RNA interference (RNAi) screen targeting 23 different components of the ESCRT machinery (ESCRT-0/I/II/III) and associated proteins. Our results highlight a role for a few selected members of this family in either the efficiency of secretion or the composition of the secreted vesicles.

RESULTS

Selected ESCRT proteins have an effect on exosome biogenesis or secretion

In order to determine whether different ESCRT members affect exosome biogenesis or secretion, we performed a medium-throughput screen in 96-well plates as previously set up in our group (Ostrowski et al., 2010). HeLa-CIITA-OVA cells (expressing MHC class II molecules and secreting ovalbumin) were infected with lentiviral vectors encoding for shRNA sequences specific for individual ESCRT proteins to invalidate each protein: 5 different shRNA sequences were used to knockdown each gene, and the control was an shRNA which does not target any human sequences (shSCR). After incubating cells for 48 h with exosome-depleted medium, exosomes from the supernatant were trapped on anti-CD63 coated beads and detected with anti-CD81 and anti-HLA-DR (MHC class II) antibodies by flow cytometry: exosomes have been shown to be enriched in MHC class II molecules and the tetraspanins CD63 and CD81 (Escola et al., 1998; Zitvogel et al., 1998); we therefore used these molecules as means of capturing and quantifying exosomes. To monitor the classical secretory pathway of soluble proteins, ovalbumin was similarly trapped on anti-OVA beads and detected with an anti-OVA antibody (Figure 1A). Finally, the efficiency of the knock-down of each gene was analyzed in exosome-secreting cells by quantitative RT-PCR (qRT-PCR). The criteria we defined for a gene to be potentially involved in exosome secretion was that at least two different shRNA sequences, which inhibited gene expression, would modulate exosome secretion in the same manner.

The results of the screen are shown in Table 1: among the 23 candidates that were analyzed, 7 were not further analyzed for the effects on exosome secretion due to the inability to demonstrate a down-regulation of the gene (VPS37B, CHMP2A/VPS2A, CHMP2B/VPS2B, CHMP4A/VPS32A, CHMP4B/VPS32B, VPS24, VPS4A). shRNA specific for four of the candidates (VPS28, VPS37A, SNF8/VPS22, VPS25) did not induce a significant modulation of exosome secretion (Supplementary Figure S1), and 5 of them (STAM2, VPS36, CHMP1B, CHMP5, CHMP6/VPS20) showed inconsistent effects of the different shRNA sequences on exosome secretion (Supplementary Figure S2).

The remaining 7 candidates reached the defined selection criteria; a second round of screening then confirmed these hits. As shown in Figure 1B, inhibiting three ESCRT-0 (HRS, STAM1) and ESCRT-I proteins (TSG101) induced a decrease in exosome secretion: 2 or more different shRNA sequences (sh1 and sh3 for HRS, sh2 and sh3 for STAM1, all shRNA for TSG101) induced at least 50% inhibition in exosome secretion. Conversely, inhibition of four ESCRT-III (CHMP4C/VPS32C), disassembly (VPS4B, VTA1/LIP5) and accessory proteins (ALIX/AIP1) induced an increase in exosome secretion (Figure 1C): at least 2 shRNA sequences (sh3 and sh5 for CHMP4C and VPS4B, sh1 and sh3 for VTA1 and ALIX)

induced an increase of 50% or more in exosome secretion as measured by our FACS-based assay.

Validation of the screen results by western blot analysis of secreted exosomes

We then chose one shRNA to validate the results of the screen by purifying exosomes from large numbers of cells (in the range of 1-2x10^8 cells) and evaluating the modification of exosome secretion by western blotting. Of the two shRNA that modulated exosome secretion in the same manner (listed in the previous section), we selected the one that induced the highest mRNA inhibition (sh3 for HRS, CHMP4C and VPS4B, sh2 for STAM1, sh1 for TSG101, VTA1 and ALIX). Exosomes were purified by differential ultracentrifugation of the supernatant of shRNA treated HeLa-CIITA-OVA cells as previously described (Thery et al., 2006), and the pellet obtained in the final 100,000 x *g* centrifugation was analyzed by western blot (Figure 2A and B). Three experiments were performed, in which we also assessed the efficient down-regulation of each target gene by qRT-PCR or western blotting of total cell lysates (Supplementary Figure S3). We analyzed by western blotting (Figures 2C, D, and E) two of the protein markers used to quantify exosomes in the FACS-based assay (MHC class II and CD63), and a chaperone described previously as enriched in exosomes secreted by mouse dendritic cells (DCs), HSC70 (Thery et al., 1999).

As compared to control cells (SCR), secretion of all markers was decreased in HRS, STAM1 and TSG101 shRNA-treated cells, which suggests a decrease in total exosome secretion by these cells. In the case of CHMP4C and VTA1 inhibition, we observed no change, or even a slight decrease, in the signal of all three markers, which does not confirm the initial conclusion from the screen, where we detected an increase in exosome secretion. The shRNA to VPS4B induced an increase in all markers as compared to exosomes from shSCR-treated cells, which indicates an increase in total secretion of exosomes, in agreement with the results from the screen. Interestingly, the inhibition of ALIX caused a clear increase in MHC class II, but not in the levels of HSC70 and CD63 as compared to control exosomes, pointing to a change in exosome composition rather than a change in exosome yield.

Description of exosomes released by shRNA-treated cells by immuno-electron microscopy

To determine whether the vesicles secreted by shRNA-treated cells displayed the same morphology as those of control cells, we then characterized exosomes by immuno-electron microscopy (immuno-EM) in a quantitative manner: the 100,000 x *g* pellets were analyzed as whole mounted samples immunogold labeled for MHC class II molecules and CD63 (Figure 3A). The diameter of 200 individual vesicles per condition as well as the amount of

gold particles of each marker per vesicle were evaluated. First, we observed differences in terms of the size of the vesicles: as shown in Figure 3B the proportion of vesicles of different sizes (30-60 nm, 60-100 nm and 100-200 nm in diameter) was slightly different in each condition. For instance, whereas shHRS increased the proportion of the smallest category (30-60 nm), inactivation of the other ESCRT components (STAM, TSG101, VTA1, VPS4B, ALIX) increased the proportion of the largest class of vesicles (> 100 nm) (Figure 3B).

We subsequently quantified the presence of CD63 and MHC class II in vesicles of different sizes. Notably, in all conditions CD63 was found to be most enriched in smaller vesicles, whereas MHC class II was detected more prominently in larger vesicles, and vesicles bearing both CD63 and MHC Class II represented on average less than 20% of the total number of vesicles (Figure 3C and supplementary Figure S4). This indicates that at least two different types of vesicles are present in the 100,000 x g pellet: one population of smaller vesicles (30-60 nm diameter) enriched in CD63, and one population of vesicles with a larger diameter (100-200 nm) and a higher MHC class II content. In addition, we observed that manipulation of ESCRT expression induced differences in the protein content of the secreted vesicles. The invalidation of VPS4B (Figure 3C), HRS or STAM1 (Supplementary Figure S4) did not strikingly alter the CD63 and MHC class II content of the different types of vesicles. By contrast, invalidation of TSG101 caused a decrease in the presence of both markers as evidenced by an increase in the proportion of vesicles devoid of CD63 and MHC class II. Conversely, the shRNA against ALIX induced a significant increase in the proportion of secreted vesicles labeled with both markers among all categories defined by their size, thus evidencing an increase in MHC class II labeling (since the proportion of CD63-positive vesicles is lower than in the control). This observation is in agreement with the previous result, highlighting by western blotting an increase in MHC class II content in shALIX-derived exosomes.

We have thus so far observed an important heterogeneity of the vesicles purified by the differential ultracentrifugation procedure classically used for exosome purification, not only in terms of size but also of protein content. Interestingly, shRNA against HRS and STAM1 both induced a general decrease of secretion of CD63 and MHC class II-bearing vesicles (as observed by western blotting) but with the former the vesicles were also of smaller size, whereas with the latter they were of larger size than the control vesicles. The most remarkable effect was observed upon invalidation of ALIX, which induced the secretion of an increased proportion of larger vesicles, and an altogether higher MHC class II content.

HeLa cells treated with shALIX have a higher MHC class II content both at the cell surface and in MVEs

In order to understand the effect induced by shALIX on exosomes secreted by HeLa-CIITA-OVA cells, we started by analyzing total cell lysates by western blotting (Figure 4A): we were able to confirm the decrease of ALIX in treated cells, and we saw an increase in MHC class II content in these cells (between 25 and 140%) as compared to control lysates; two reference genes, HSC70 and Actin (according to the experiment), displayed similar levels in control and treated cells.

Next, we performed a surface FACS staining to detect MHC class II and CD63 at the plasma membrane (Figure 4B): the former was notably increased in shALIX-treated cells as revealed by the quantification of five experiments, whereas the levels of tetraspanin CD63 were not significantly modified as compared to control shSCR-treated cells.

We then processed shSCR- and shALIX-treated cells for immuno-EM: ultrathin cryosections were labeled for MHC class II and CD63, and the labeling of each protein was quantified in the MVEs. As shown in Figure 4C, the labeling of MHC class II molecules was higher in the MVEs of cells invalidated for ALIX, unlike the staining for CD63 in these compartments, which was unaltered as compared to the control cells.

We have thus observed a general increase of MHC class II in cells upon ALIX inactivation, in total cell lysates, and both at the cell surface (according to FACS results) and in intracellular compartments such as MVEs (as shown by the quantification by immuno-EM).

The invalidation of ALIX induces an increase in MHC class II content in human immature DCs, but not in secreted exosomes

We next asked if the increase in MHC class II induced by shALIX, in the cells as well as in exosomes, could be reproduced in cells that endogenously synthesize and express MHC class II molecules. We decided to focus on human monocyte-derived DCs, since the exosomes they secrete can induce immune responses and are thus clinically relevant (Viaud et al., 2011; Zitvogel et al., 1998). Monocytes were purified from blood of healthy donors, transduced with shSCR or shALIX, and differentiated to DCs with IL-4 and GM-CSF (in the presence of puromycin, to eliminate cells that were not transduced) for 5 days. Both untreated cells, and cells treated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) to induce maturation, were analyzed by western blotting: as shown in Figure 5A, an effective down-regulation of ALIX (between 60 and 100%) was observed in shALIX-treated cells. MHC class II content was significantly increased in shALIX-treated cells compared to control cells, but only in immature DCs, not in LPS+ IFN-γ treated cells. The result obtained with mature cells is expected since the maturation process leads to an induction of MHC class II

synthesis as well as a decrease in MHC class II endocytosis and degradation (Guermonprez et al., 2002), thus causing a substantial increase in MHC class II content both at the cell surface as well as in total, which is unlikely to be further augmented by other treatments.

The surface staining of MHC class II by flow cytometry (Figure 5B, top panel) was consistent with the western blotting: we observed an increase in shALIX-treated and immature cells as compared to shSCR immature DCs, but no differences between LPS+IFN-γ treated cells transduced with control or ALIX shRNA. It is important to note the presence of two peaks in untreated shSCR cells, which represent immature and spontaneously mature cells (probably due to their manipulation in culture); in untreated shALIX cells, however, only one peak, intermediate between both peaks in the control condition, is visible. This either suggests that shALIX DCs are in a state halfway between immature and mature, or that the increase in MHC class II due to the treatment with the shRNA is such that it cannot be higher after the maturation process. Interestingly, we also observed a slight increase in the % of cells expressing high level of surface CD86 in untreated DCs from 4 out of 7 donors, transduced with shALIX (data not shown). CD86 is used to monitor maturation of DCs; thus, the increase in MHC class II labeling can be partly due to the maturation of a fraction of the cultured cells in the shALIX condition. Similarly to what was previously seen with HeLa-CIITA cells, CD63 content at the surface of dendritic cells was not modified in shALIX cells as compared to the control, regardless of the maturation status (Figure 5B, bottom panel).

We also performed the FACS-based assay used in the screen to see if we could induce in DCs a similar increase in the detection of CD63-MHC class II-CD81-positive exosomes as with HeLa-CIITA cells after shALIX treatment (see Figure 1B); in this case only CD63-coated beads were used since we wished to detect secreted exosomes but not OVA. Figure 5C shows the results obtained with immature DCs treated with shALIX from seven different donors (all results are expressed as percent of the corresponding shSCR, which corresponds to 100%). We were unable to observe a significant increase in the signal measured with the FACS-based assay: only 2/7 experiments (donors 3 and 6) showed a slight increase, albeit much lower (less than 50%) than the one found with HeLa-CIITA cells (around 75-100%). The other experiments showed no effect (donor 4), or a decrease of 50% or higher (donors 1, 2, 5 and 7). These results suggest that ALIX is involved in the trafficking of MHC class II molecules in different cell types, and might also have an effect on the sorting of molecules into exosomes, at least in the HeLa-CIITA model.

DISCUSSION

The original studies in reticulocytes (Harding et al., 1983; Pan et al., 1985), those in EBV-B lymphocytes (Raposo et al., 1996), as well as the more recent ones using the HeLa-CIITA cell model (Ostrowski et al., 2010) support an endosomal origin of exosomes. Since they correspond to the internal vesicles of MVEs, candidate targets for intervention on their biogenesis and secretion are mechanisms supposed to be involved in cargo sequestration at the endosomal membrane and vesicle budding and scission. The discovery that the ESCRT machinery was involved in MVE formation (Henne et al., 2011; Raiborg and Stenmark, 2009) opened a new avenue to interfere with the formation of ILVs and thereby exosome secretion. However the evidence that ESCRT-independent mechanisms also operate for the biogenesis of MVEs (Theos et al., 2006; Trajkovic et al., 2008) suggested that these mechanisms could be broader and therefore more complex and redundant that initially envisioned.

In this study we set out to investigate for the first time the involvement of a large panel of ESCRT components (23 genes in total) in exosome biogenesis and secretion in HeLa-CIITA cells. For this purpose, we performed a medium-throughput screening using a FACS-based assay, which allows relative quantification of CD63-, CD81- and MHC class II-bearing exosomes secreted in the supernatant of cells treated with small-interfering short-hairpin RNA. Since exosomes have been previously described as enriched in tetraspanins and MHC class II molecules, we designed this assay to quantify the population of vesicles bearing CD63 and either CD81 or MHC class II, which we assumed would correspond most specifically to vesicles originating from late endosomes. Our immuno-EM analysis of exosomes secreted by control cells revealed that the preparations contain not only vesicles double positive for CD63 and MHC class II, but also vesicles bearing only MHC class II or CD63, and vesicles that appear negative for both markers (but maybe express low amounts that are under the limits of detection by immuno-EM). Exosomes positive for MHC class II and CD63 corresponded to 10-20% of all secreted vesicles in most conditions employed in this study. It is likely that other exosomal markers such as CD81 also have a heterogeneous distribution in exosomes, and consequently the first read out of the screen is likely to provide information on a selected fraction of all secreted exosomes. Nevertheless, the results are then further validated by additional complementary methods. We employed western blotting to evaluate the decrease or increase on protein components as a consequence of the invalidation of different genes, we analyzed quantitatively by immuno-EM the size related to protein content of isolated exosomes, and for some components we have investigated the nature of the compartments *in situ* in the cells.

Our assays together highlight and extend previous reports and descriptions (Bobrie et al., 2012; Fevrier and Raposo, 2004) that cells secrete a heterogeneous population of exosomes with different sizes and composition. This fully corroborates the observations that cells host different MVEs and that within MVEs, ILVs are not all similar in morphology and composition (Fevrier et al., 2004; Mobius et al., 2003; White et al., 2006). Our recent studies demonstrated the existence of different subpopulations of exosomes in preparations of membrane vesicles purified by differential ultracentrifugation from mouse mammary carcinoma 4T1 cells (Bobrie et al, 2012). Thus, proteins specifically enriched in the different subpopulations of vesicles will have to be identified in the future, to allow development of other capture-based assays to identify the molecular machinery involved in their respective secretion.

In our screen some targeted ESCRT components did not have an impact on exosome secretion (VPS28, VPS37A, SNF8/VPS22, VPS25), despite inducing a clear decrease in RNA expression. Inactivation of ESCRT-II subunits did not change the pattern of secretion. These observations are in line with the findings that ESCRT-II, as compared to other ESCRT complexes, does not appear to be involved in HIV budding (Langelier et al., 2006) or in EGF-receptor degradation (Bowers et al., 2006). However, we cannot exclude, as discussed by Malerod and colleagues (Malerod et al., 2007), that to obtain an effective inactivation of ESCRT-II, it is necessary to concomitantly inactivate at least 2 of the 3 subunits. Inactivation of ESCRT-III subunits (CHMP1B, CHMP5, CHMP6/VPS20) did not consistently decrease or increase vesicle secretion, except in the case of CHMP4C, which appeared to induce an increase in exosomes measured in the cell-conditioned medium, but we were unable to reproduce these results with the characterization of purified exosomes. One possible explanation is that although ESCRT-III was shown to be involved in vesicle budding and scission in cell-free studies (Wollert and Hurley, 2010), inactivation of these components in cells does not lead to a clear reduction of MVEs (Bache et al., 2006), and our unpublished results). These MVEs are maybe unable to fuse with lysosomes (Bache et al., 2006, Simoes et al., in revision) but they could still fuse with the plasma membrane to release exosomes. Moreover, it should be noted that different isoforms of ESCRT-III components exist in cells so we cannot exclude a redundancy in their function.

In contrast to ESCRT-II and -III, inactivation of components of ESCRT-0 (HRS, STAM1) and ESCRT-I (TSG101) as well as more late acting components (VTA1, VPS4B) and the accessory protein ALIX evidenced consistent alterations of exosome secretion. Previous studies on an oligodendrocytic cell line secreting the proteolipid PLP in association with exosomes highlighted a formation and release process independent of ESCRT function but requiring the synthesis of the lipid ceramide by a type II sphingomyelinase (Trajkovic et al., 2008). In this study, inactivation of Hrs, TSG101, Alix and VPS4 did not alter consistently

the sequestration of PLP in ILVs of MVEs, although it reduced EGF transport to the MVEs. However, our data indicate that secreted exosomes are heterogeneous on the basis of size and cargo, and in this previous study only one population has been followed (i.e. PLP exosomes) which renders difficult to conclude that all exosome populations are ESCRT-independent. Our results indicate that loss of function of selected ESCRT components, which in some cases decreases (HRS, STAM1, TSG101) or increases (VPS4B, ALIX) the amount of vesicles and cargo associated with vesicles, also modulates the nature and protein contents of the vesicles. Inactivation of HRS, STAM1 and TSG101 decreased the amount of vesicles and associated proteins (MHC Class II, CD63) but the nature of the vesicles that were still present in the medium was different for the 3 components. Upon HRS inactivation, while vesicles with sizes comprised between 60-100, 100-200 nm largely decreased, a population of smaller vesicles (30-60 nm) was increased as compared to control cells. These observations are consistent with the findings by Tamai and colleagues showing that Hrs is required for facilitating the secretion of exosomes in mouse DCs, although in this latter work, Hrs-dependent exosome secretion was induced by ovalbumin or a calcium ionophore, rather than spontaneous as in our HeLa-CIITA cells (Tamai et al., 2010). Although also part of the ESCRT-0 complex, the inactivation of STAM rather decreased the population of vesicles with diameters comprised between 30-60 and 60-100 nm while larger vesicles were clearly more abundant. We cannot exclude that part of these vesicles are not endosomal-derived exosomes but rather larger vesicles that could be derived from the plasma membrane, and that STAM inactivation increases their budding and release. Inactivation of TSG101 resulted in an increase in the release of larger vesicles similar to that observed with the loss of function of STAM, but these vesicles showed a different protein composition compared to the other conditions, as evidenced by an increase in vesicles negative for both CD63 and MHC class II in all size categories.

Thus, interfering with the function of some early components of the ESCRT machinery decreases exosome production but the nature of the vesicles that are still present in the supernatants is different. This is consistent with the model that ESCRTs act sequentially in cargo sequestration and vesicle formation, which consequently impacts on endosome maturation including the formation of ILVs of endosomes. Indeed, Razi and Futter have shown that the effects of HRS inactivation are different from TSG101 loss of function during the maturation of MVEs (Razi and Futter, 2006). Unexpectedly, inactivation of ALIX and VPS4B increased exosome secretion as monitored during the screen but also after evaluation of the amount of proteins associated with exosomes. However, whereas inactivation of VPS4B increased all markers associated with exosomes (CD63, MHC class II, HSC70), inactivation of ALIX only increased the amount of MHC class II while CD63 and HSC70 remained unchanged as compared to control cells. In the absence of ALIX, medium-

sized and larger vesicles (60-100/100-200 nm) were mostly released. These vesicles were enriched in MHC class II, indicating that ALIX is likely to selectively control the composition of exosomes. A recent report shows that ALIX is required for the sequestration of syndecans within exosomal membranes via its interaction with syntenin (Baietti et al., 2012). Our results do not challenge these findings but rather put forward the idea that, depending on the cell types and the cargoes associated with different exosome subpopulations, the mechanisms involved may slightly change, and that a tight balance of mechanisms operates at the endosomal membrane, controlling protein sorting to distinct ILVs subpopulations that may in some cases have different fates (van Niel et al., 2011). It would be important in the future to elucidate the molecular requirements for these different exosomal cargoes within the same cell type.

Our observations that the inactivation of ALIX increased an MHC class II-positive exosome subpopulation led us to further investigate at the total cell level the alterations of MHC class II expression by FACS and immuno-EM on ultrathin cryosections. Extending the observations of the increase in MHC class II in exosomes, FACS analysis clearly indicated a consistent increase of MHC class II at the cell surface (but not of CD63) and immuno-EM revealed numerous MVEs densely labeled for MHC class II. Therefore, ALIX depletion increases MHC class II expression not only in exosomes but also in the cells. These observations suggest that ALIX may be directly or indirectly required for MHC class II degradation/downregulation. Such increased expression of MHC class II is similar to the one recently reported upon CD63 silencing (Hoorn et al., 2012). Given that ALIX interacts with syntenin (Baietti et al., 2012), a CD63 interacting protein (Latysheva et al., 2006), we may propose that ALIX also allows CD63-dependent MHC class II degradation in HeLa CIITA and dendritic cells. It would be of interest to investigate whether MHC class II interacts with ALIX as it has been shown for the Transferrin receptor (TfR) (Geminard et al., 2004). ALIX interacts with a consensus motif on the TfR cytoplasmic domain and this interaction promotes TfR stabilization and trafficking to the exosomal pathway.

In monocyte-derived human DCs, similarly to HeLa-CIITA cells, an increase of MHC class II at the cellular level upon inactivation of ALIX was observed suggesting that ALIX may be involved in the trafficking and degradation of MHC class II molecules in other cell types as well. Nevertheless, the FACS-based exosome capture assay failed to evidence increased secretion of CD63- and MHC class II- or CD81-bearing exosomes by ALIX-depleted DCs. On the opposite, a decreased secretion in the majority of donors was observed (Figure 6C). This could suggest either that exosomes secreted by ALIX-inactivated DCs did not present an increase in MHC class II at their surface, or that secretion of CD63-bearing exosomes was decreased in ALIX-impaired DCs, which was compensated by the increased amount of MHC class II on these exosomes. These hypotheses must now be

addressed by analyzing the relative representation of each subtype of extracellular vesicles (CD63+, MHC class II+, and CD63+/MHC II+) in the exosomes secreted by DCs, with or without ALIX inactivation, and the morphology and content of MVEs in these cells. In any case, the difference between DCs and MHC II-expressing HeLa might imply that ALIX impacts on MHC class II trafficking and/or exosome biogenesis but that different mechanisms are exploited by these very different cell types.

Our findings altogether reinforce the concept that cells host different subpopulations of MVEs and secrete a heterogeneous population of exosomal vesicles. Whereas ESCRT-independent mechanisms are involved in MVE/exosome biogenesis (Trajkovic et al., 2008), our observations and recent reports (Baietti et al., 2012; Tamai et al., 2010) show that the formation and secretion of exosomes may also rely on ESCRT function, and that only selected components may be involved in protein sorting and the formation of the intralumenal vesicles fated to be secreted as exosomes. These observations will certainly contribute to our understanding of the mechanisms involved in exosome formation and will open an avenue for modulating the nature of exosomes and their composition.

MATERIALS AND METHODS

Cells and reagents

HeLa-CIITA OVA cells (subclone B6H4, Ostrowski et al., 2010) were cultured in DMEM containing 4.5 g/L glucose (Life Technologies) 10% fetal calf serum (FCS, Gibco or PAA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco), 300 μg/mL hygromycin B (Calbiochem) and 500 μg/mL geneticin (Life Technologies).

Lentiviruses expressing shRNA and a puromycin resistance gene were obtained from the RNAi consortium (TRC) and produced as described previously (Moffat et al, 2006). As a control, the scrambled sequence of shRNA to GFP (not targeting any human gene) was used (shSCR). All shRNA sequences are indicated in Supplementary Table 1. Sequences of shRNA used for large scale cultures were: HRS (sh3) 5′-CCAGCCTTACAACATGCAGAA-3′; STAM (sh2) 5′-CCCTTTC-CACTTTGTATCCAA-3′; TSG101 (sh1) 5′-CCCAGAATAAGTTATTGCAGT-3′; CHMP4C (sh3) 5′- GCAGAATAAGCGAGCTGCATT -3′; VPS4B (sh3) 5′-CGAAGATTTGAGAAACGAATT-3′; VTA1 (sh1) 5′-GCCTGGAAACTTGGTGCTTTA-3′; ALIX (sh1) 5′-GCTGCTAAAC-ATTACCAGTTT-3′.

Antibodies used for FACS analysis were: mouse anti-human CD63 (clone FC-5.01, Zymed; or clone H5C6, BD Biosciences) and goat anti-OVA (ICN) for coupling to beads; PE-conjugated anti-HLA-DR (clone L243, BD Biosciences), anti-CD81 (clone JS-81, BD Biosciences) and rabbit anti-OVA (Sigma) coupled to Alexa488 according to the manufacturer's instructions (DyLight 488 Antibody Labeling Kit, Pierce). Antibodies used for Western blotting were: mouse anti-TSG101 (Genetex); rat anti-HSC70 (Stressgen); mouse anti-CD63 (Zymed), anti-HLA-DR (clone 1B5), rabbit anti-VPS4B (Abcam), goat anti-HRS (Santa Cruz) and rabbit anti-VTA1 (Abcam); HRP-conjugated secondary antibodies (Jackson Immunoresearch). Antibodies used for immunofluorescence were: mouse anti-CD63 (Zymed), mouse anti-HLA-DR (L243); Alexa488-anti-mouse and Cy3-anti-rabbit (Molecular Probes); DAPI (Molecular Probes). Antibodies used for immunoelectron microscopy were: rabbit anti-HLA-DR (H. Ploegh), mouse anti-HLA-DR (L243) and mouse anti-CD63 (Zymed).

Screening procedure

HeLa-CIITA OVA cells were plated in 96-well plates at 2,500 cells per well the day before infection. Triplicate wells of cells were infected with lentiviral vectors in the presence of 8 μg/mL polybrene (Sigma); puromycin (Gibco) was added at 5 μg/mL 36 h later. Medium was replaced with exosome-depleted medium (complete medium depleted of FCS-derived exosomes by overnight centrifugation at 100,000 x *g*) after 36 h. Supernatants were

collected 48 h later for OVA and exosome quantification, and the amount of live cells in each well was determined using the Cell titer blue reagent viability assay (Promega). OVA and exosome secretions per well were normalized to the number of viable cells, and only wells containing at least 75,000 cells were analyzed.

Aldehyde-sulfate beads (4 μm, Life Technologies) were coated with anti-human CD63 or anti-OVA antibodies by incubating 140 μg of antibody with 70 μL beads overnight at room temperature, followed by blocking of remaining activated groups by performing three washes with PBS-4% BSA. Beads were stored in 500 μL of PBS-0,1% glycine at 4°C.

Cell culture supernatants were cleared by centrifugations at 300 x *g* for 5 min and 2,000 x *g* for 20 min. Cleared supernatants (100 μL) were incubated with 0,1 μL anti-CD63- and 0,1 μL anti-OVA-coupled beads in round-bottom 96-well plates, overnight at 4°C with 7,500 rpm horizontal shaking. Beads were washed twice in PBS-0.5% BSA and incubated with PE-anti-HLA-DR (1:50), PE-anti-CD81 (1:100) and Alexa488-anti-OVA (1:200) for 30 min on ice. Beads were washed in PBS-0.5% BSA and acquired on a MACSQuant flow cytometer (Miltenyi Biotec). Data were analysed with FlowJo software (Tree Star). Negative staining was obtained with beads incubated with exosome-depleted medium, and for each culture condition, arbitrary units (AU) of exosome or OVA secretion were calculated as a percentage of positive beads x mean fluorescence intensity (MFI) of positive beads. Three independent experiments were performed, in which shSCR and each gene-specific shRNA were analyzed in triplicate wells. In each experiment, the basal level of exosome or OVA secretion (control) was calculated as the mean of AU of triplicate wells transduced with control scrambled shRNA (shSCR), and the AU of each shRNA-expressing cells was expressed as percent of control. The mean value of triplicates from three independent experiments (n=9) is shown in Figures 1, S1, and S2.

RNA extraction and real-time qPCR

RNA was extracted from cells in each well with the RNA XS kit (Macherey Nagel) at the end of each experiment, quantified with a NanoDrop spectrophotometer (Thermo Scientific) and 200 ng were reverse transcribed with AMV Reverse Transcriptase (Finnzymes). cDNA (1/20) was used for the PCR reaction, performed in triplicates with SYBRgreen (Thermo Scientific), on a Lightcycler LC480 (Roche). Primers were obtained from Qiagen (QuantiTect Primer Assay). Cycle threshold (Ct) for each gene was normalized to Ct for *GAPDH*, and results are expressed as percentage of control cells (shSCR).

Alternatively, RNA was extracted from 10^6 cells with the RNA II kit (Macherey Nagel).

Exosome purification

For each condition 5×10^7 cells were plated in exosome-depleted medium and incubated for 48h. Supernatants were then harvested and exosome purification was performed as previously described (Thery et al, 2006). Supernatants were centrifuged at 300 x g for 5 min to eliminate floating cells, at 2,000 x g for 20 min to remove cell debris, and at 10,000 x g for 40 min to separate microvesicles. A final ultracentrifugation step at 100,000 x g for 1,5 h was performed to obtain exosomes, followed by a washing step in PBS to eliminate contaminating proteins. Exosomes were resuspended in 100 µL PBS, and protein content was measured by MicroBCA (Pierce) in the presence of 2% SDS.

Western blotting

Total protein extracts were obtained from transduced cells by incubating 4×10^6 cells in 100 µL lysis buffer: 50 mM Tris-HCl pH 7.4, 30 mM NaCl, 0,5% Triton X-100, 0,1% sodium azide, and a cocktail of protease inhibitors (Roche), for 30 min on ice. Lysates were centrifuged for 5 min at 15,000 x g at 4°C. Supernatants were collected and total protein concentration was measured by MicroBCA (Pierce), with 2% SDS.

A volume of exosomes secreted by 30×10^6 cells was loaded on 4-12% Bis-Tris gels (Life Technologies). Alternatively, 100 µg of total cell lysate were used for western blotting. Proteins were transferred onto an Immobilon PVDF membrane (Millipore), and incubated with primary antibodies followed by the corresponding secondary HRP-conjugated antibodies. Revelation was performed with the BM Chemiluminescence Blotting Substrate kit (Roche Applied Science). Images were acquired using ChemiDoc XRS (BioRad). Alternatively, after incubation with primary antibodies, the membranes were incubated with Cy5-conjugated secondary antibodies, and images were acquired on a Typhoon FLA 9000 scanner (GE Healthcare). Band intensity was quantified with ImageJ software (NIH).

Flow cytometry analysis

For surface staining, cells were harvested and incubated with anti-MHC class II or CD63 antibody (2 µg/mL) for 30 min on ice, followed by anti-mouse-Alexa488 antibody (1/200) for 20 min on ice, and acquisition on a MACSQuant flow cytometer.

For total staining, after a surface staining cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences). Cells were then washed with Perm/Wash buffer (BD Biosciences), incubated with primary and secondary antibodies, and analyzed by flow cytometry.

Electron microscopy

Exosomes resuspended in PBS were deposited on formvar-carbon coated copper/palladium grids for 20 min at RT, fixed for 20 min in PBS-2% PFA (Electron Microscopy Sciences), and quenched in PBS 50 mM glycine. Grids were incubated with mouse anti-MHC class II followed by protein A-gold 15 nm (PAG15, CMC Utrecht). After fixation in glutaraldehyde 1% (Electron Microscopy Sciences) grids were incubated with anti-CD63, followed by PAG10. After a final fixation step in glutaraldehyde, grids were washed and contrast was performed with an ice-cold mixture of methylcellulose and uranyl acetate for 15 min. Grids were air-dried before observation of the samples.

Cells transduced with lentiviruses were fixed in 0.2M phosphate buffer at pH 7.4 with 2% PFA and 0,125% glutaraldehyde for 2 h at RT. Cells were then processed for ultrathin cryosectioning as previously described (Raposo et al, 2001), and labeled with rabbit anti-MHC class II (1/200) or anti-CD63 (2 μg/mL) antibodies, followed by PAG15 and PAG10, respectively.

All samples were observed at 80kV with a CM120 Twin Philipps electron microscope (FEI Company) and digital images were acquired with a numeric camera (Keen View; Soft Imaging system). Quantification was performed with the iTEM software (Soft imaging System). For exosome quantifications, one image obtained by multiple image acquisition (comprising nine individual images) was analyzed. Labeling of MHC class II and CD63 in MVEs was quantified in 50 compartments from different images.

Dendritic cell transduction and FACS assay

Monocyte-derived dendritic cells were obtained from blood samples of healthy donors. This study was conducted according to the Helsinki Declaration, with informed consent obtained from the blood donors, as requested by our Institutional Review Board. PBMC were isolated by density gradient centrifugation (LymphoPrep, Axis-Shield), CD14^{+} monocytes were obtained by magnetic sorting (Miltenyi Biotec) and cultured in RPMI 1640 (Gibco) supplemented with 10% FCS (Biowest), 50 mM 2-ME, 10 mM HEPES, 100 IU/mL penicillin and 100 μg/mL streptomycin (Gibco), with 50 ng/mL IL-4 and 100 ng/mL GM-CSF (Miltenyi Biotec).

Monocytes were transduced with shSCR or shALIX according to the protocol by Satoh and Manel (Satoh and Manel, 2012). Briefly, HEK293T cells were transfected with plasmids pLKO.1, pΔ8.9, pCMV-VSV-G and TransIT-LT1 transfection reagent (Mirus Bio) to generate a lentiviral vector carrying an shRNA sequence and the puromycin resistance gene; or with plasmids pSIV3[+] and pCMV-VSV-G to generate helper particles containing the Vpx protein which overcomes the blocking of lentiviral infections normally observed in monocyte-derived

DCs (Goujon et al, 2006). Fresh monocytes were incubated with equal volumes of both particles and 8 μg/mL protamine for 48h, and incubated with fresh medium with 2 μg/mL puromycin for 72h. Immature dendritic cells thus obtained were counted and 2×10^5 cells per well were incubated in exosome-depleted medium for 24h (in triplicates) to detect secreted exosomes by trapping on beads followed by staining for flow cytometry, as described above. Maturation was induced by treating DCs with LPS (1 µg/mL, Sigma) and IFN-γ (2500 IU/mL, Miltenyi Biotec). Cells were also incubated with antibodies against CD14, CD1a, CD80, CD86, HLA-DR (1/100, BD Biosciences) and CD63 (1/100, BioLegend) together with the corresponding isotype controls (1/100, BD Biosciences) to check DC differentiation and maturation status.

Statistical analysis

Statistical tests were performed with GraphPad Prism software (GraphPad Software, Inc.). One-way ANOVA followed by Tukey's post test was used to analyze screening results, and a paired t-test was done to analyze western blot or FACS results. Values are shown as mean ± s.d. in figures 1, 2, S1 and S2, or as median in figures 2, 4, 5 and S3.

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FIGURE LEGENDS

Figure 1: Modulation of exosome secretion by members of the ESCRT family as evidenced by the screening of a library of shRNA. **A**) Exosomes and ovalbumin (OVA) in cell-conditioned medium were measured by trapping each on a separate antibody-coated bead, staining with antibodies with different fluorophores, and analyzing the beads by flow cytometry. Exosome secretion (top panel), ovalbumin secretion (middle panel) and gene downregulation (bottom panel) are shown for genes whose inactivation induced a decrease in exosome secretion (**B**), or an increase in exosome secretion (**C**). Results are shown as percentage of the control (shSCR) arbitrary units (AU), mean ± s.d. of three experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%), n.d. = not done. One-way ANOVA followed by Tukey's post test, * p< 0,05, ** p< 0,01, *** p< 0,001

Figure 2: Validation of the screening. Exosomes were purified by differential centrifugations and characterized by western blotting. **A, B**. Exosomes secreted by 30×10^6 cells were characterized by western blotting, using three different markers enriched on exosomes (MHC class II, HSC70 and CD63). Quantification of the signal of MHC class II (**C**), HSC70 (**D**) and CD63 (**E**) was performed with the ImageJ software; results are expressed as % of the control (shSCR) AU; the red bar indicates the median, and the black bar highlights the level of the control (shSCR, 100%).

Figure 3: Analysis of exosomes secreted by the different shRNA-treated cells by immuno-electron microscopy. **A**. Whole-mount exosomes were stained for MHC class II (15 nm gold particles) and CD63 (10 nm gold particles). Both the number of gold particles as well as the diameter (measured with the iTEM software) were quantified for 200 vesicles per condition. Images are shown for shSCR and shALIX derived exosomes. **B**. A quantification of the number of small (30-60 nm in diameter), intermediate (60-100 nm diameter) and large (100-200 nm diameter) vesicles is shown for each condition (top graphs). **C**. Results of the quantification are illustrated here for each condition (shSCR, shVPS4B, shALIX, shTSG101) as percentage of vesicles with no gold particles (CD63- MHC II-), only 10 nm gold particles (CD63+), only 15 nm gold particles (MHC II+), or both gold particles (CD63+ MHC II+), for small (30-60 nm), intermediate (60-100 nm) and large (100-200 nm) particles. Bar 100 nm.

Figure 4: Characterization of shSCR and shALIX-treated cells. **A**. Western blotting of total cell lysates (100 μg/lane); staining with antibodies against ALIX, MHC class II and ACTIN is shown. Quantifications of six independent experiments (right panel) show the down

regulation of ALIX and the increase of MHC class II in shALIX treated cells as compared to the control, and no difference for reference proteins HSC70 or ACTIN; results are expressed as % of control AU and the red bar indicates the median. **B**. FACS analysis of control and shALIX treated cells (top panel), and graph of the MFI of the markers MHC class II and CD63. Results are shown as percentage of the control MFI for four or five independent experiments (bottom panel). **C**. Immuno-electron microscopy of ultrathin cryosections of shSCR (top left panel) or shALIX (top right panel) treated cells, with staining for MHC class II (15 nm gold) and CD63 (10 nm gold). The number of MHC class II (bottom left panel) or CD63 (bottom right panel) gold particles quantified on 50 individual MVEs for each condition is shown, the red bar indicates the median. Bar 100 nm.

Paired t-test, n.s. not significant, ** $p < 0,01$, *** $p < 0,001$.

Figure 5: Characterization of shSCR and shALIX-treated human primary dendritic cells. **A**. Western blotting of total cell lysates from untreated DCs or LPS+IFN-γ treated DCs; staining with antibodies against ALIX, MHC class II and HSC70 are shown. Quantifications of band signal performed on western blots of cell lysates from seven donors (right panel) show the down-regulation of ALIX and the increase of MHC class II in shALIX treated cells as compared to the control. Results are expressed as % of AU of the untreated control (Untreated shSCR) **B**. FACS analysis of control and shALIX treated cells with markers MHC class II (top) and CD63 (bottom) and graph of the MFI expressed as percentage of the control (immature peak in the case of untreated shSCR; MFI marker – MFI isotype control) for six donors; untreated DCs (left panel), or LPS+IFN-γ treated DCs (right panel). **C**. Exosome secretion by DCs from seven donors: exosomes were quantified by trapping on anti-CD63 coated beads and staining with anti-CD81 and anti-HLA-DR antibodies. Results are expressed as percentage of the control (shSCR) AU, mean ± s.d. of one experiment performed in triplicates for each donor. The black horizontal bar indicates the level of the control (shSCR, 100%). Paired t-test, n.s. not significant, * $p < 0,05$, *** $p < 0,001$

Figure S1: No modulation of exosome secretion by the inactivation of members of the ESCRT family. Exosome secretion (top panel), ovalbumin secretion (middle panel) and gene downregulation (bottom panel) are shown for genes whose inactivation had no effect on exosome secretion. Results are shown as percentage of the control (shSCR) AU, for three experiments performed in triplicates. One-way ANOVA followed by Tukey's post test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$

Figure S2: Genes identified as inducing discrepant effects on exosome secretion when inactivated. Exosome secretion (top panel), ovalbumin secretion (middle panel) and gene downregulation (bottom panel) are shown for genes whose inactivation had discrepant effects on exosome secretion between the different shRNA sequences. Results are shown as percentage of the control (shSCR) AU, for three experiments performed in triplicates. One-way ANOVA followed by Tukey's post test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$

Figure S3: Gene downregulation in lysates of shRNA-treated cells. Western blotting (shHRS, shVPS4B, shVTA1, shALIX) or real-time quantitative PCR (shSTAM1, shTSG101, shCHMP4C) were performed to check for protein or mRNA downregulation. Results from three independent experiments are shown for each condition, and expressed as percentage of the control (shSCR) AU, the red bar indicates the median. One representative western blot is shown for four of the genes.

Figure S4: Analysis of exosomes secreted by the different shRNA-treated cells by immuno-electron microscopy. **A**. Whole-mount exosomes were stained for MHC II (15 nm gold particles) and CD63 (10 nm gold particles). Both the number of gold particles as well as the diameter (measured with the iTEM software) were quantified for 200 vesicles per condition. Images are shown for shSCR and shALIX derived exosomes, as well as graphs showing the number of gold particles and the diameter per vesicle (each dot on the graph represents a vesicle). Bar 100 nm. **B**. Results of the quantification are illustrated here for four conditions (shSCR, shHRS, shSTAM1, shVTA1) as percentage of vesicles with no gold particles (CD63- MHC II-), only 10 nm gold particles (CD63+), only 15 nm gold particles (MHC II+), or both gold particles (CD63+ MHC II+), for small (30-60 nm), intermediate (60-100 nm) and large (100-200 nm) particles.

Table 1 – Screening results

Most shRNA modulate exosome secretion	Most shRNA have no effect on exosome secretion	Discrepant effects of the different shRNA in terms of exosome secretion	Inability to demonstrate inhibition of the gene
HRS	VPS28	STAM2	VPS37B
STAM1	VPS37A	VPS36	CHMP2A
TSG101	SNF8	CHMP1B	CHMP2B
CHMP4C	VPS25	CHMP5	CHMP4A
VPS4B		CHMP6	CHMP4B
VTA1			VPS24
ALIX			VPS4A
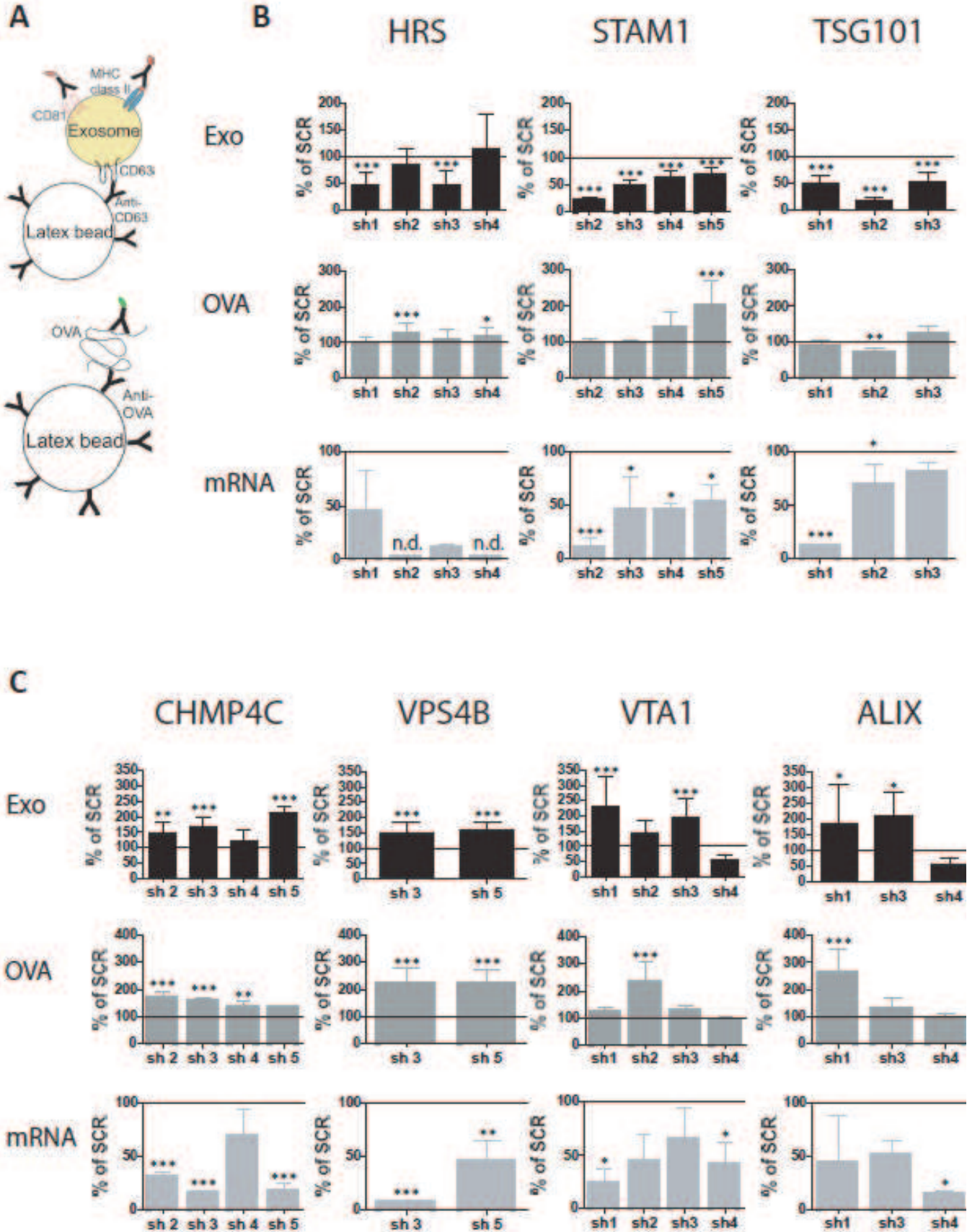
FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

A

CELL LYSATES

shSCR shALIX

ALIX — 96 kDa

MHC class II α chain — 31 kDa

ACTIN — 42 kDa

ALIX
***

MHC II
**

HSC70
n.s.

ACTIN
n.s.

B

MHC II

CD63

— Isotype
— shSCR
— shALIX

***

n.s.

C

shSCR   MHC II 15
         CD63   10

shALIX MHC II 15
         CD63   10

MHC II

CD63

FIGURE 5

A CELL LYSATES

Untreated DCs   LPS+IFN-γ DCs

SCR   ALIX   SCR   ALIX

ALIX — 96 kDa

MHC class II — 31 kDa

HSC70 — 70 kDa

ALIX

MHC II

B

Untreated DCs

LPS + INF-γ DCs

MHC II

CD63

— Isotype
— shSCR
— shALIX

C Exosome secretion by untreated DCs (FACS-based assay)

FIGURE S1

FIGURE S2

FIGURE S3

CELL LYSATES

A

HRS

STAM1 mRNA

TSG101 mRNA

B

CHMP4C mRNA

VPS4B

VTA1

ALIX

FIGURE S4

A

shSCR — MHC II 15 / CD63 10

shALIX — MHC II 15 / CD63 10

MHC II

CD63

B

shSCR

shHRS

shSTAM1

CD63+ MHCII+
MHC II+
CD63+
CD63- MHCII-
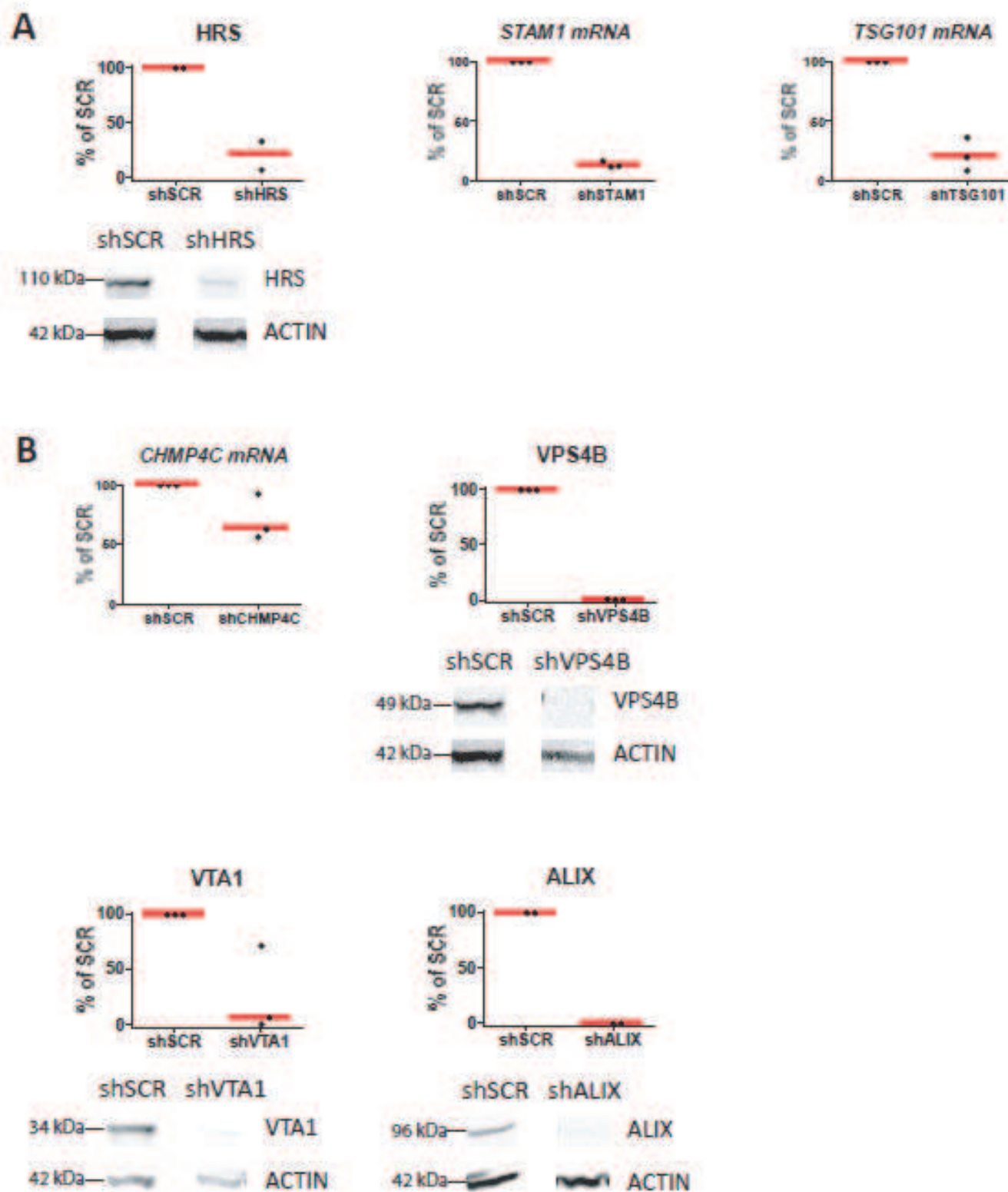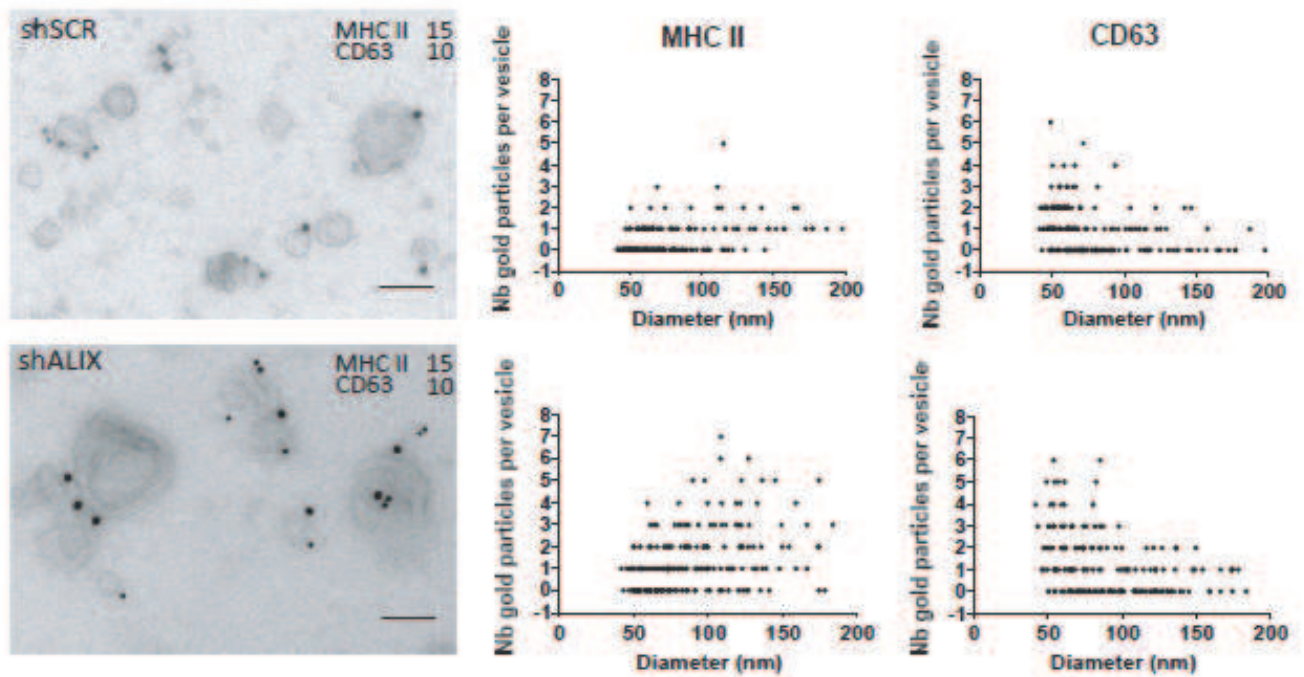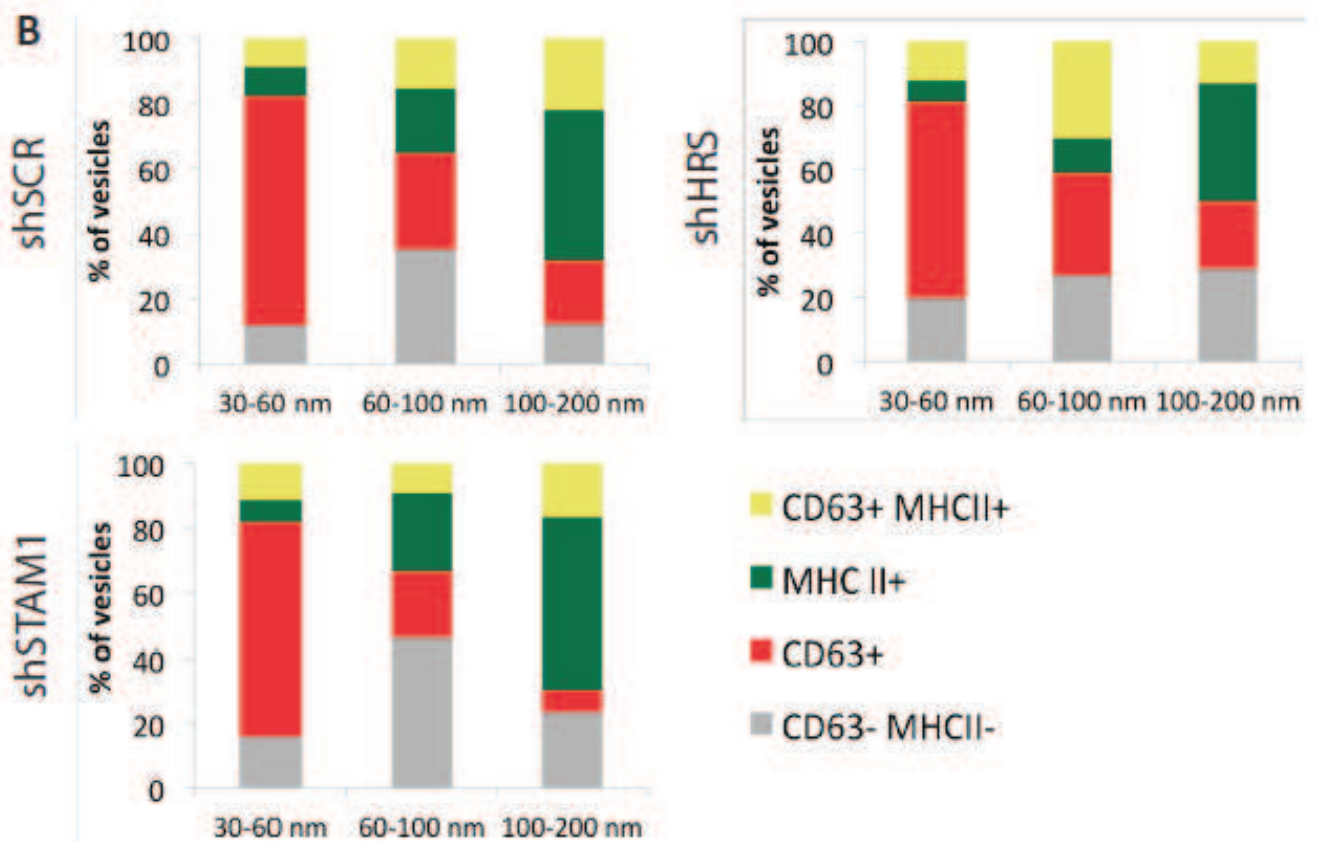
Supplementary Table 1 – shRNA sequences

	Gene symbol	shRNA	Clone ID	Sequence
ESCRT-0	HRS (HGS)	sh1	TRCN0000037894	CCGCATGAAGAGTAACCACAT
		sh2	TRCN0000037895	CCTGTACTCTTCACCTGTGAA
		sh3	TRCN0000037896	CCAGCCTTACAACATGCAGAA
		sh4	TRCN0000037897	CGTCCGTAACAAGATCCTGTA
		sh5	TRCN0000037898	GCACGTCTTTCCAGAATTCAA
	STAM1	sh1	TRCN0000004061	CTAGTGAAGTAAGCAACGTAT
		sh2	TRCN0000004062	CCCTTTCCACTTTGTATCCAA
		sh3	TRCN0000004063	TGTGTATCAAACTGTGGCAAA
		sh4	TRCN0000004064	CCAGGTGCCAAACTATAACTT
		sh5	TRCN0000004065	CTGCTAAATGCCACTGACAAT
	STAM2	sh1	TRCN0000056773	GCACGGAAAGTGAGAGCTTTA
		sh2	TRCN0000056774	GCACTGGACAAGACACTGTTT
		sh3	TRCN0000056775	GCACCAGTGTACTCAGTCTAT
		sh4	TRCN0000056776	CTACAGAAGATTGGAGTCTTA
		sh5	TRCN0000056777	GCCTGCTCAAACTTCATATTT
ESCRT-I	TSG101 (VPS23)	sh1	TRCN0000007560	CCCAGAATAAGTTATTGCAGT
		sh2	TRCN0000007561	CGTCCTATTTCGGCATCCTAT
		sh3	TRCN0000007562	CCTTATCTACATGAATGGAAA
		sh4	TRCN0000007563	GCCTTATAGAGGTAATACATA
		sh5	TRCN0000007564	GCAGAGCTCAATGCCTTGAAA
	VPS28	sh1	TRCN0000004148	GCGGTGGTGAAGACAATGCAA
		sh2	TRCN0000004149	GAGGGAGAAGTACGACAACAT
		sh3	TRCN0000004150	CTCGCTCTTCATCACGGTCAT
		sh4	TRCN0000004151	GTCAGCCTACAACGCCTTCAA
		sh5	TRCN0000010854	AGAGCTGTTTGCGGTGGTGAA
	VPS37A	sh1	TRCN0000073183	CCTGGAAACATGAACTGCCAA
		sh2	TRCN0000073184	CCGACAAAGATGACTTAGTAA
		sh3	TRCN0000073185	GCCAATTTCATGCTCCACTAT
		sh4	TRCN0000073186	CGACATCACTTAATGGATAAA
		sh5	TRCN0000073187	ACCATAAACAACCTGACAATT
	VPS37B	sh1	TRCN0000122091	CAGAATGTTCAGCTTAACAAA
		sh2	TRCN0000139237	CCAGGTTCTCTTTGAAGCCTA
		sh3	TRCN0000140011	GCCGATGAAGTGGATGCAATT
		sh4	TRCN0000140578	GAGACCCTGTTAGCACTTCTT
		sh5	TRCN0000144349	CCTGAAGGTTTCTATGAAGAA
ESCRT-II	SNF8 (VPS22)	sh1	TRCN0000015723	CGCTCGTTCAGTAAGCATAAT
		sh2	TRCN0000015724	GCCATCGCCAAGAAGAAACTT
		sh3	TRCN0000015725	CTGATAACTTTGGAGGAACTA
		sh4	TRCN0000015726	GTGAGATCAAAGCCAGTCTTA
		sh5	TRCN0000015727	GCCCAGGATGTCAGTCAAGAT
	VPS25	sh1	TRCN0000122421	GAGATCATCACTGTCAGCGAT
		sh2	TRCN0000141204	CCCTTTACTTCTTACCTCCCA
		sh3	TRCN0000143104	CATATGTCTGTCCCTTGGATA

		sh4	TRCN0000144276	CTTCTTTACGTTACAACCGAA
		sh5	TRCN0000144757	GAGTCGATCCAGATTGTATTA
	VPS36	sh1	TRCN0000128378	GATGAGACCATCAGGTTTAAA
		sh2	TRCN0000128720	CGTCATGGTAATTGAGCTTCA
		sh3	TRCN0000129882	GCAAACTAATGATCAAGGCTA
		sh4	TRCN0000130011	GAGTAGTAAGAACTCCTACAT
		sh5	TRCN0000146785	CAGAGGAAATGACACAAAGAA
ESCRT-III	CHMP1B	sh1	TRCN0000158735	GATTTCTGCTTTGATGGACAA
		sh2	TRCN0000160848	GACCATGAATCTGGAGAAGAT
		sh3	TRCN0000160950	GACAAATTCGAGCACCAGTTT
		sh4	TRCN0000166491	CCAGAACCAAGTGGATATGCT
		sh5	TRCN0000166391	CAACATGGAAGTTGCGAGGAT
		sh6	TRCN0000161212	GCACTCTTAGCTGGATTCTAA
		sh7	TRCN0000159294	GCTGGATTCTAAAGTTCTGTA
		sh8	TRCN0000166094	GAACTGAGTAGGAGTGCCAAA
		sh9	TRCN0000161474	GCGACATTGAAGACCATGAAT
		sh10	TRCN0000164819	CAGAAGGGCAACATGGAAGTT
	CHMP2A	sh1	TRCN0000122604	GCCAAGCAAGGCCAGATGGAT
		sh2	TRCN0000139572	CATTGATGATGCCATGGGTGA
		sh3	TRCN0000140813	GCAGGCAGAGATCATGGATAT
		sh4	TRCN0000143644	CATGAACAGACAGCTGAAGTT
		sh5	TRCN0000143825	GCTATGTGCGCAAGTTTGTAT
	CHMP2B	sh1	TRCN0000128433	GCAGTTAACAAGAAGATGGAT
		sh2	TRCN0000129922	GCAGCTTTAGAGAAACAAGAA
		sh3	TRCN0000130815	GCCTTAAATAGCACAGACCTA
		sh4	TRCN0000130897	GCTTGACACCTGCCTTAAATA
		sh5	TRCN0000131086	GCTTACCATCTGCCTCTACTT
	VPS24 (CHMP3)	sh1	TRCN0000297794	CAAAGTCTTGTGAAGATTCCA
		sh2	TRCN0000280247	GTGAAACGATCTGTGAAAGAT
		sh3	TRCN0000280248	CAATGAGTGGTCATTGAAGAT
		sh4	TRCN0000297792	GATCAGGAAGAAATGGAGGAA
	CHMP4A	sh1	TRCN0000148217	GACAGAGAAGATACTGATCAA
		sh2	TRCN0000148454	CACAAACTGACGGGACATTAT
		sh3	TRCN0000148490	CAAGAAGTATGGGACCAAGAA
		sh4	TRCN0000148705	CCTTTCTTCCTTAAGTGCCAA
		sh5	TRCN0000150154	GAGCTAAAGGAGGATAACTTT
	CHMP4B	sh1	TRCN0000147769	GTTAAGCAAGAAACAGGAGTT
		sh2	TRCN0000148126	GATGAGTTAATGCAGGACATT
		sh3	TRCN0000150181	GCAGTTGAAATGACCTGAAAT
		sh4	TRCN0000180330	CGGCACATTATCAACCATCGA
	CHMP4C	sh1	TRCN0000179569	GATGGCACACTTTCTACCATT
		sh2	TRCN0000146312	CCAAGAAATCTCAGAAGCATT
		sh3	TRCN0000180929	GCAGAATAAGCGAGCTGCATT
		sh4	TRCN0000147799	GCGATGAAATCTGTTCATGAA
		sh5	TRCN0000179242	GACAAATATCCGCCTTCCAAA
	CHMP5 (Vps60)	sh1	TRCN0000163206	GAGTTGGATGCACTAGGTGAT
		sh2	TRCN0000161107	GAGGATTTACAAGACCAGCTA
		sh3	TRCN0000163175	GAAGGCATACAAGCCAGTGAA
		sh4	TRCN0000159946	GAATCCATTGACAAGAAGATT
		sh5	TRCN0000161287	GATGCTATGAAACTGGGAGTA

		sh6	TRCN0000158654	CTGGATGAAGATGATTTAGAA
		sh7	TRCN0000162096	CAACATGGACAGGCCATTATA
		sh8	TRCN0000164404	CCAGTGAAGATCGACCAGATT
		sh9	TRCN0000161695	GACAATCTTGCCAACAGTCAT
	CHMP6 (VPS20)	sh1	TRCN0000151037	GCAATCACTCAGGAACAAATA
		sh2	TRCN0000152315	CCAGATCGAAATGAAAGTGAT
		sh3	TRCN0000154612	GCTGCTCAAGAAGAAGCGATA
		sh4	TRCN0000155050	GCTCAAGAAGAAGCGATACCA
		sh5	TRCN0000155260	GCGCAATCACTCAGGAACAAA
ATPase complex	VPS4A	sh1	TRCN0000162998	GCAAAGAGAAACACGGCAAGA
		sh2	TRCN0000163363	GCCGAGAAGCTGAAGGATTAT
		sh3	TRCN0000161666	GTGTCGTTTAGATCAGGGTAA
		sh4	TRCN0000159521	CGAGAAGCTGAAGGATTATTT
		sh5	TRCN0000163870	CCTCCACGCTATCAAGTATGA
	VPS4B	sh1	TRCN0000021734	GCGGTCACTATCTAACACAAA
		sh2	TRCN0000021735	GCACTGAAAGAGGCTGTGATA
		sh3	TRCN0000021736	CGAAGATTTGAGAAACGAATT
		sh4	TRCN0000021737	CCATTGTTATAGAACGACCAA
		sh5	TRCN0000021738	GCTGATCCTAACCATCTTGTA
	VTA1 (LIP5)	sh1	TRCN0000072688	GCCTGGAAACTTGGTGCTTTA
		sh2	TRCN0000072689	CCTTCTATACTGCAAGTCTTT
		sh3	TRCN0000072690	GCACAGGTGTAGCAAGTAATA
		sh4	TRCN0000072691	GAAGGCAACATACATCCATAA
		sh5	TRCN0000072692	GCAAATATGCTGGCAGTGCTT
Accessory proteins	ALIX (AIP1)	sh1	TRCN0000029394	GCTGCTAAACATTACCAGTTT
		sh2	TRCN0000029395	CCTGAATTACTGCAACGAAAT
		sh3	TRCN0000029396	CCAGAACAAATGCAGTGATAT
		sh4	TRCN0000029397	GCAGAACAGAACCTGGATAAT
		sh5	TRCN0000029398	GCATCTCGCTATGATGAATAT

3) Function of other molecules, especially SNARE proteins, in exosome biogenesis or secretion

a) SNAREs and other molecules

Summary

In order to assess the potential role of other selected proteins in exosome biogenesis and/or secretion, we performed our RNA interference screening on HeLa-CIITA cells, which targeted SNARE proteins and several other components described in the literature as potentially involved in exosome biogenesis or secretion. Among the SNAREs, two promoted exosome secretion (STX2 and SEC22L3), and three instead prevented it (YKT6, STX1, GOSR1). Among the other candidates, inhibition of TSAP6 and ATP6V0a2 decreased exosome secretion (in accordance with their previously proposed effect in other mammalian or invertebrate models), whereas inhibition of SMPD3 (neutral sphigomyelinase), and two other isoforms of the V-ATPase (ATP6V0a1 and ATP6V0a3) increased exosome secretion, suggesting that they may function in preventing secretion.

Background

SNAREs proteins are involved in the final steps of fusion of membranes for intracellular transport to occur between organelles (Jahn and Scheller, 2006). Some of these components are thus highly likely to be involved in the fusion of MVEs with the plasma membrane during the release of exosomes, although the specific SNARE complex responsible for this process has yet to be discovered.

In addition, several other molecules have been described in the literature as possible mediators of exosome biogenesis: neutral sphingomyelinase 2 (nSMase2 or SMPD3) (Trajkovic et al., 2008), citron kinase (CIT) (Loomis et al., 2006), the brefeldin A (BFA)-inhibited guanine nucleotide-exchange protein BIG2 (Islam et al., 2007), diacyl glycerol kinase α (DGKA) (Alonso et al., 2007), annexin A2 (ANXA2, which has been found in exosomes by proteomic approaches) (Thery et al., 2001); and secretion: V-ATPase (ATP6V0a isoforms) (Liegeois et al., 2006), TSAP6 (Lespagnol et al., 2008; Yu et al., 2006) and phospholipase D2 (PLD2) (Laulagnier et al., 2004a).

FIGURE 14 - No modulation of exosome secretion by the inactivation of members of the SNARE family.
Exosome secretion (top panel) and ovalbumin secretion (bottom panel) are shown for genes classified as R-SNARE (**A**), SNARE-like (**B**), and Q-SNARE (**C**) whose inactivation had no effect on exosome secretion. Results are shown as percentage of the control (shSCR) arbitrary units (AU), or percentage of the plate mean, as indicated, for three experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%). One-way ANOVA followed by Tukey's post test, * p< 0,05, ** p< 0,01, *** p< 0,001.

We have thus tested the involvement of these molecules by means of an RNA interference screen.

Materials and methods

The screen was performed according to the protocol described in the manuscript "ESCRTs differentially modulate exosome biogenesis, composition and secretion in MHC class II-expressing cells". The different shRNA sequences employed are listed in Appendix I - Table 4.

Results and discussion

Among the 21 SNARE genes that were analyzed, eleven failed to show any effect on the secretion of exosomes (as mentioned in the previous section, exosome detection in the cell conditioned medium involved trapping on CD63-coated beads, and staining with antibodies against MHC class II and CD81): the R-SNAREs VAMP3 and VAMP7, the SNARE-like protein SEC22L2, and the Q-SNAREs STX3A, STX5A, STX8, STX17, BET1L, VIT1A, VIT1B and GOSR2 (**Figure 14**). Another five genes corresponding to R-SNARES displayed discrepant effects of the different shRNA sequences on exosome secretion: STX4A, STX6, STX7, STX12 and BNIP1 (**Figure 15**). Finally, five proteins have emerged as potential candidates in regulating the secretion of exosomes: YKT6 (R-SNARE), STX1 and GOSR1 (Q-SNARES) induce a significant increase in exosome secretion upon invalidation, whereas the knockdown of SEC22L3 (SNARE-like protein) and STX2 (Q-SNARE) induces a decrease (significant but weak) in exosome secretion (**Figure 16**). Interestingly, these results are not consistent with the a previously reported role for VAMP7 in the release of exosomes by K562 cell line (Fader et al., 2009), nor with the SNARE complex involved in the exocytosis of lysosomes by normal rat kidney (NRK) cells, which could also be required for the exocytosis of other intracellular compartments such as MVEs and includes VAMP7, syntaxin 7 (STX7) and synaptotagmin 7 (Rao et al., 2004). They are, however, in line with another report (Proux-Gillardeaux et al., 2007), in which expression of the Longin domain of VAMP7 impairs the secretion of late endosomes and lysosomes, without affecting the secretion of exosomes by MDCK cells.
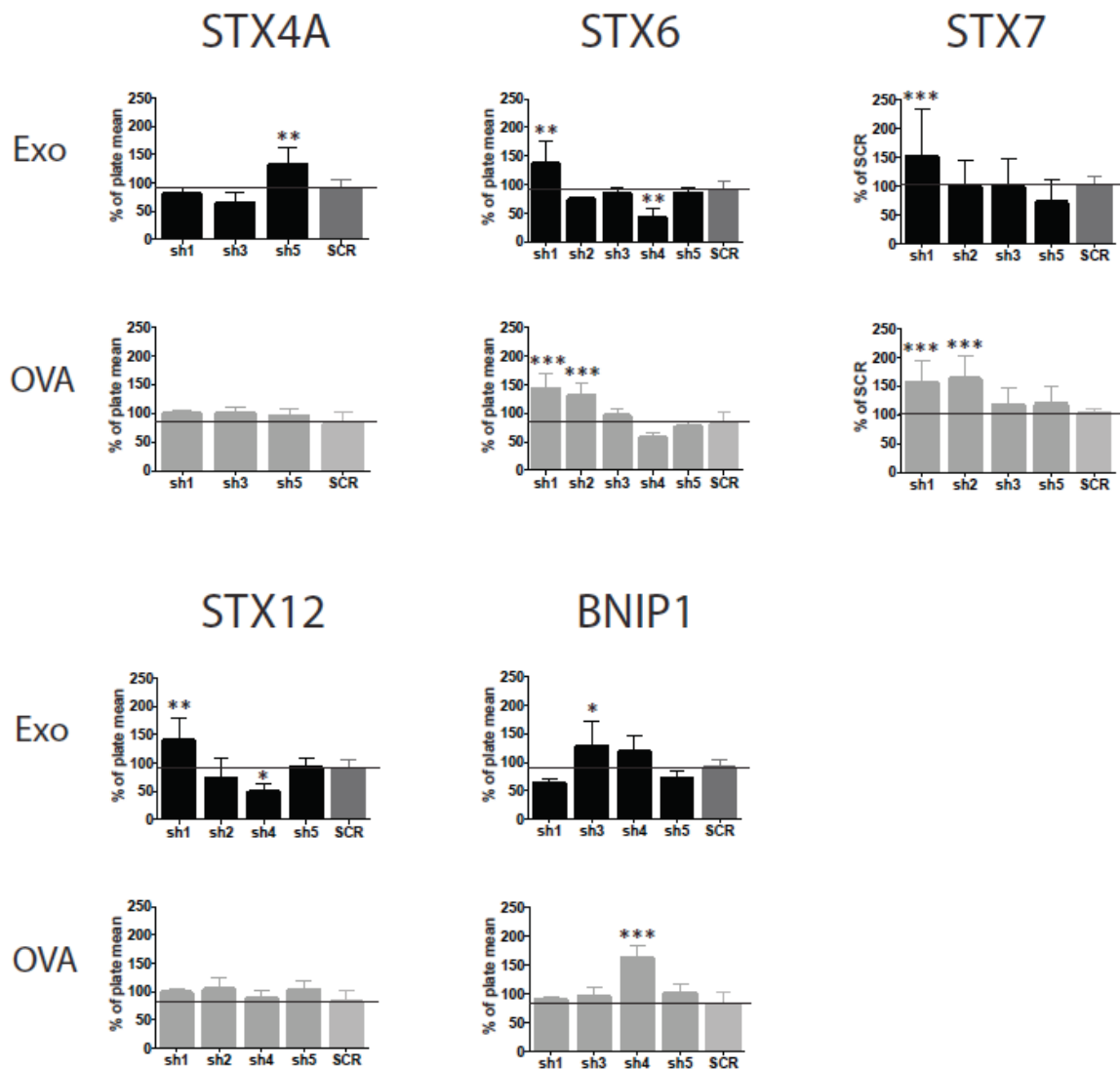
FIGURE 15 - Genes from the SNARE family (Q-SNARE) identified as inducing discrepant effects on exosome secretion when inactivated.

Exosome secretion (top panel) and ovalbumin secretion (bottom panel) are shown for genes whose inactivation had discrepant effects on exosome secretion between the different shRNA sequences. Results are shown as percentage of the control (shSCR) AU, or percentage of the plate mean, as indicated, for three experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%). One-way ANOVA followed by Tukey's post test, * p< 0,05, ** p< 0,01, *** p< 0,001.

FIGURE 16 - Modulation of exosome secretion by members of the SNARE family as evidenced by the screening of a library of shRNA.

Exosome secretion (top panel) and ovalbumin secretion (bottom panel) are shown for genes classified as whose inactivation induced an increase (**A**) or decrease (**B**) in exosome secretion. Results are shown as percentage of the control (shSCR) AU, or as percentage of the mean value of the entire 96-well plate, as indicated; mean ± s.d. of three experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%). One-way ANOVA followed by Tukey's post test, * p< 0,05, ** p< 0,01, *** p< 0,001.

The analysis of other proteins potentially involved in the exosome secretion pathway showed the lack of effect of PLD2, ANXA2, and the V-ATPase subunit ATP6V0a4, while DGKA, BIG2 and CIT evidenced discrepant effects of the different shRNA sequences (**Figure 17**). Other candidates induced an increase in exosome secretion when inactivated, such as SMPD3, and the V-ATPase subunits ATP6V0a1 and ATP6V0a3, whereas the invalidation of TSAP6 and ATP6V0a4 caused a decrease in exosome secretion (**Figure 18**). The results obtained by inhibition of TSAP6 are in agreement with previous reports (Amzallag et al., 2004; Lespagnol et al., 2008; Yu et al., 2006). The ATP6V0a2 subunit of the v-ATPase has revealed results that seem to be in line with previous studies in the worm *C. elegans* (Liegeois et al., 2006), although the conflicting results obtained with two other subunits might imply that the regulation carried out by the V-ATPase is more complex in humans. Surprisingly, the inactivation of the neutral sphyngomyelinase II (SMPD3) has shown contradictory results to a report that indicates a reduced exosome secretion upon nSMase invalidation (Trajkovic et al., 2008). This could potentially be due to the use of different cell lines in each study (HeLa-CIITA vs. a murine oligodendroglial cell line) that might not use the same molecular machineries for exosome biogenesis. Also, both studies follow the fates of different populations of exosomes (one associated to PLP, whereas the other is identified by the simultaneous presence of CD63, MHC class II and/or CD81), which might rely on different pathways for their biogenesis.

Nevertheless, these candidates require further study to confirm their function, and in particular the confirmation of a significant downregulation of the gene induced by the different shRNA sequences, with the use of qRT-PCR or western blotting.

Conclusion

Some proteins other than the ESCRT machinery seem to be involved in the exosome secretion pathway in HeLa-CIITA cells, either during the biogenesis or the release of these vesicles, and they impact on exosome yield in different ways.
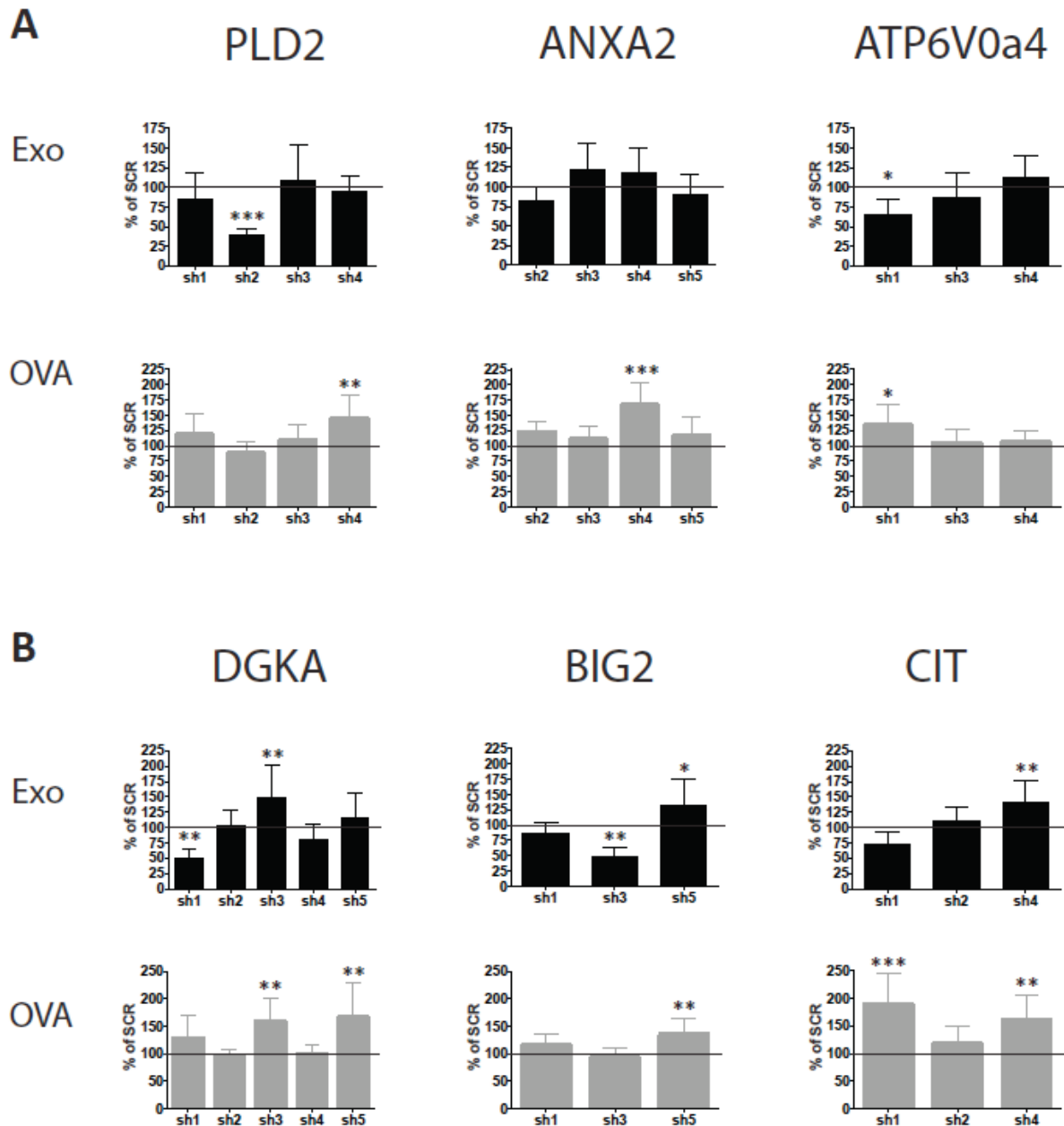
FIGURE 17 - Genes identified as not modulating exosome secretion (A**), or inducing discrepant effects on exosome secretion when inactivated (**B**).**

Exosome secretion (top panel) and ovalbumin secretion (bottom panel) are shown for genes whose inactivation had discrepant effects on exosome secretion between the different shRNA sequences. Results are shown as percentage of the control (shSCR) AU, or as percentage of the plate mean, as indicated, for three experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%). One-way ANOVA followed by Tukey's post test, * p< 0,05, ** p< 0,01, *** p< 0,001.
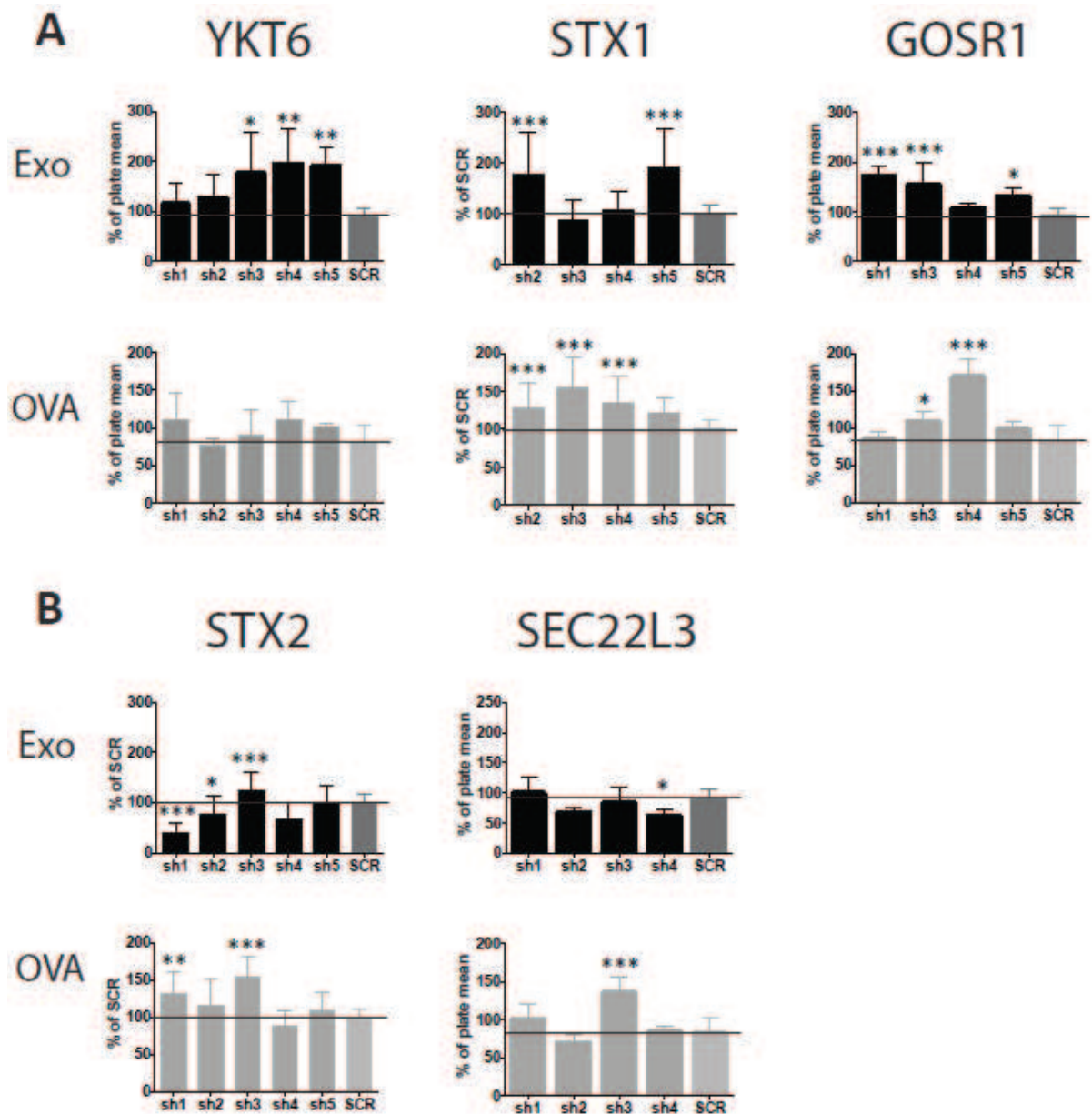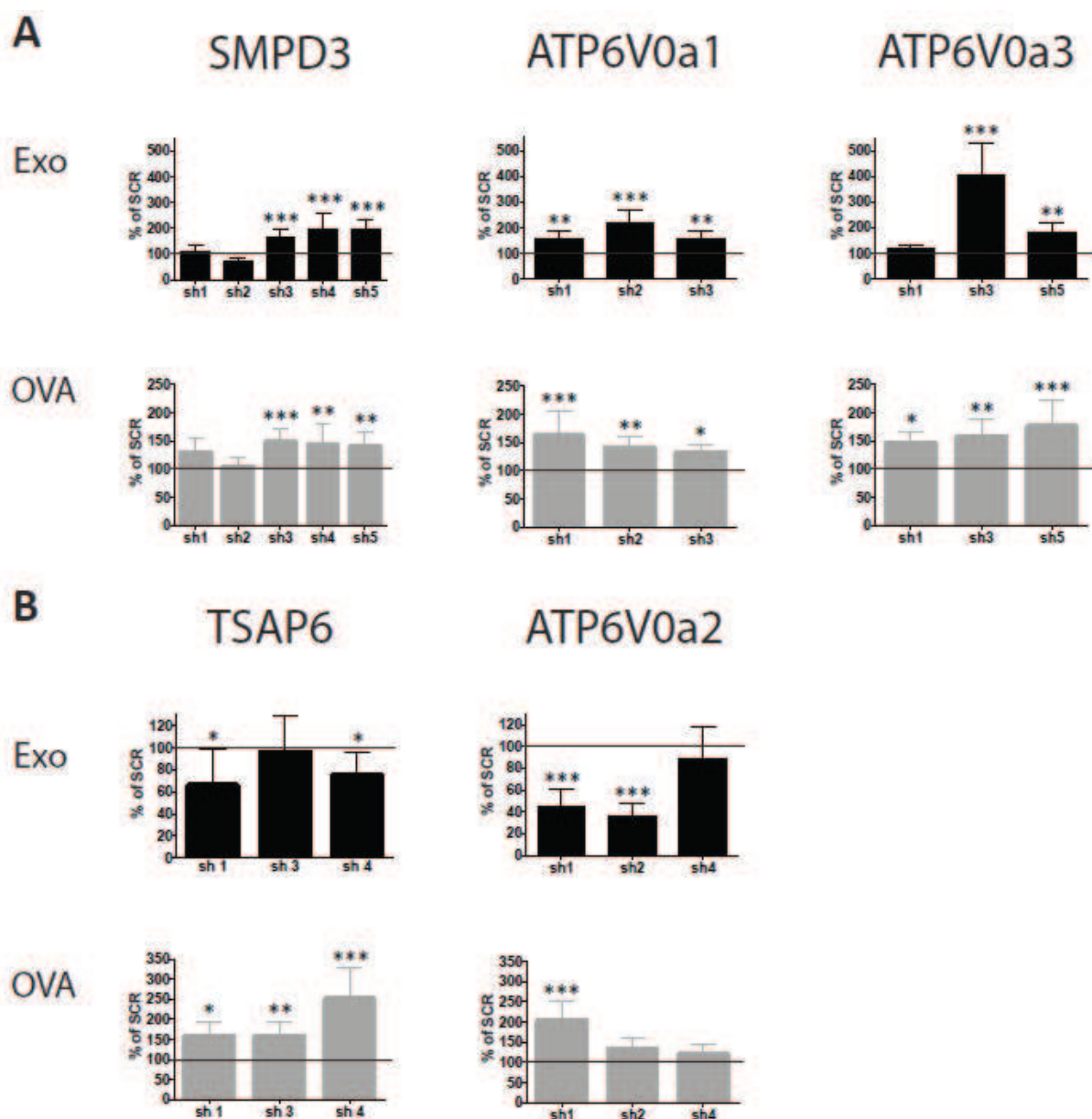
FIGURE 18 - Modulation of exosome secretion by genes as evidenced by the screening of a library of shRNA.

Exosome secretion (top panel) and ovalbumin secretion (bottom panel) are shown for genes whose inactivation induced an increase in exosome secretion (**A**), or a decrease in exosome secretion (**B**). Results are shown as percentage of the control (shSCR) arbitrary units (AU), or as percentage of the plate mean, as indicated, mean ± s.d. of three experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%), n.d. = not done. One-way ANOVA followed by Tukey's post test, * p< 0,05, ** p< 0,01, *** p< 0,001.

b) RAB5 isoforms

Summary

Due to reports of functional differences between three isoforms of the GTPase RAB5, we employed the RNA interference screen to assess the role of the individual isoforms in exosome secretion. As previously observed for RAB5A (Ostrowski al, 2010) we confirmed a decrease in exosome secretion by cells invalidated for RAB5A, but also for RAB5B. By contrast, invalidation of RAB5C lead to an increased exosome secretion. We also analyzed the effect of these isoforms by purifying exosomes and characterizing them by western blotting and immuno-EM.

Background

RAB5 is a small GTPase that localizes to early endosomes, and is involved in their homotypic fusion, as well as in the heterotypic fusion of endocytic vesicles and early endosomes *in vitro* (Gorvel et al., 1991; Stenmark, 2009). RAB5 has three highly related isoforms, RAB5A, B and C, which have been shown to cooperate in the regulation of homotypic fusion of early endosomes (Bucci et al., 1995). A study from the group of P. Stahl showed that RAB5C knockdown had little effect on EGFR degradation, as opposed to RAB5A and B, which inhibited the degradation of the EGFR and delayed its trafficking from early to late endosomes (Chen et al., 2009). Thus, RAB5 isoforms may have different functions in a given cell type. The screening performed in our lab to unravel the functions of Rab proteins on exosome secretion (Ostrowski et al., 2010) showed that inhibition of RAB5A decreased exosome secretion, whereas that of RAB5C increased it (our unpublished results). We thus wished to extend these finding to the other RAB5 isoform, and study the secreted exosomes in greater detail.

Materials and methods

The following protocols are described in the manuscript "ESCRTs differentially modulate exosome biogenesis, composition and secretion in MHC class II-expressing cells": Screening procedure, Exosome purification, Western blotting, Electron microscopy, and Cells and

A

RAB5A RAB5B RAB5C

Exo

OVA

B EXOSOMES

MHC class II   HSC70   CD63

CELL LYSATES

RAB5A — 25 kDa
ACTIN — 42 kDa

RAB5B — 25 kDa
ACTIN — 42 kDa

RAB5C — 26 kDa
ACTIN — 42 kDa

C EXOSOME ANALYSIS BY IMMUNO-EM

CD63+ MHCII+
MHC II+
CD63+
CD63- MHCII-

SCR   RAB5A   RAB5C

30-60 nm 60-100 nm 100-200 nm
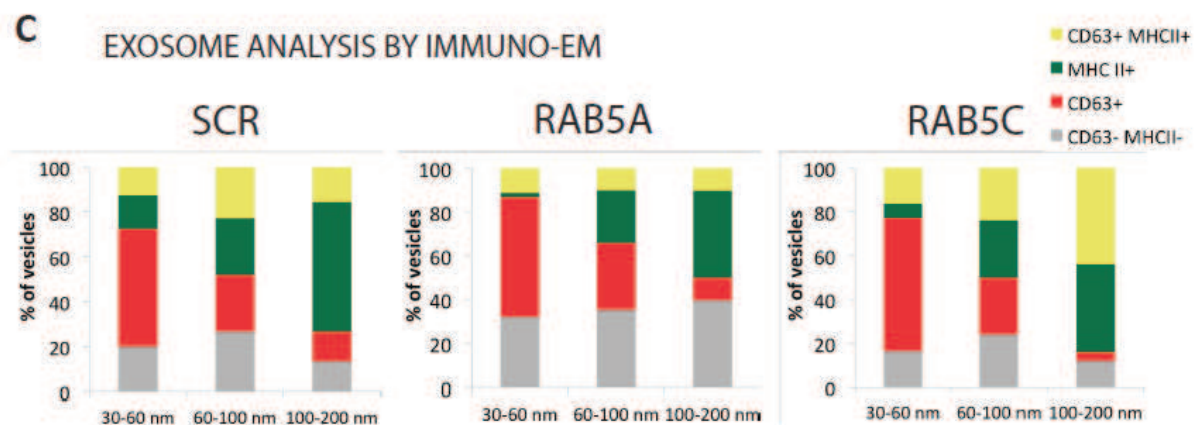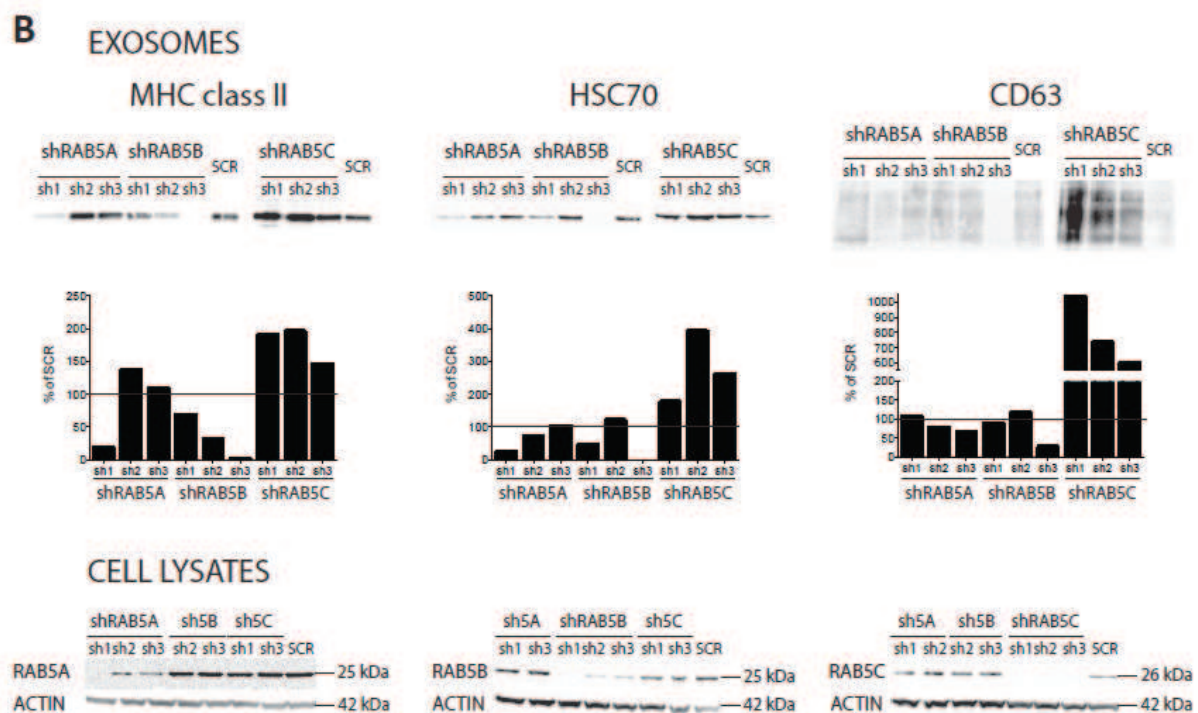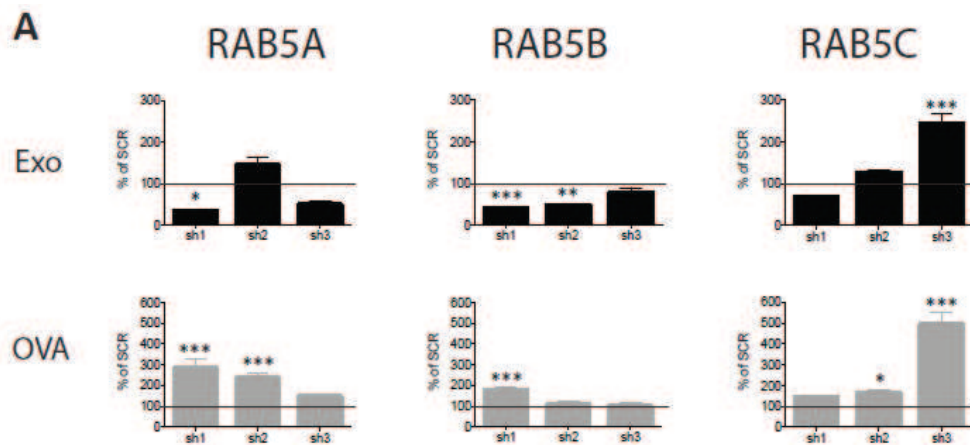
FIGURE 19 - Differential effects of RAB5 isoforms on exosome secretion.

A) Decrease in exosome secretion induced by RAB5A and RAB5B, and increase in exosome secretion induced by RAB5C, as evidenced by a FACS -based assay performed with three shRNA sequences per gene. Exosome secretion (top panel) and ovalbumin secretion (bottom panel) are shown for the three isoforms. Results are shown as percentage of the control (shSCR) AU, for two experiments performed in triplicates. The black horizontal bar indicates the level of the control (shSCR, 100%). One-way ANOVA followed by Tukey's post test, * $p < 0,05$, ** $p < 0,01$, *** $p < 0,001$ **B**) Exosomes secreted by 30×10^6 cells from one experiment were purified by differential ultracentrifugation and characterized by western blotting, using three different markers enriched on exosomes (MHC class II, HSC70 and CD63). Quantification of the signal of MHC class II, HSC70 and CD63 was performed with the ImageJ software; results are expressed as % of the control (shSCR) AU, and the black horizontal bar indicates the level of the control (shSCR, 100%). Below, western blots for total cell lysates (100 μg) were performed to check for RAB5A, RAB5B or RAB5C protein downregulation. **C**) Analysis of exosomes secreted by the different shRNA-treated cells by immuno-electron microscopy: whole-mount exosomes were stained for MHC class II (15 nm gold particles) and CD63 (10 nm gold particles). Both the number of gold particles as well as the diameter (measured with the iTEM software) were quantified for 200 vesicles per condition. Results of the quantification are illustrated here (for SCR, RAB5A sh1 and RAB5C sh3) as percentage of vesicles with no gold particles (CD63- MHC II-), only 10 nm gold particles (CD63+), only 15 nm gold particles (MHC II+), or both gold particles (CD63+ MHC II+), for small (30-60 nm), intermediate (60-100 nm) and large (100-200 nm) particles.

reagents. In addition to the reagents described in the latter, these antibodies were used: monoclonal anti-RAB5A, polyclonal rabbit anti-RAB5B (provided by P. Stahl), and polyclonal rabbit anti-RAB5C (Sigma). The different shRNA sequences employed are listed in Appendix I - Table 4.

Results and discussion

We first assessed the effect of shRNA directed against the RAB5 isoforms on exosome secretion, as evidenced by the FACS-based assay described earlier. The invalidation of RAB5A and B by at least 2 different shRNA induced a decrease in exosome secretion, whereas the opposite was true upon inhibition of RAB5C (**Figure 19A**).

Subsequently, exosomes were purified by differential ultracentrifugation from cell conditioned media, and characterized by western blotting with the use of three exosomal markers (MHC class II, HSC70 and CD63). Cell lysates were also processed by western blotting to evaluate the downregulation of each isoform (**Figure 19B**), and showed an important decrease of the protein targeted by the specific shRNA, without affecting the levels of the other two isoforms: the inhibition of gene expression was highest with sh1 from RAB5A and B, and equally as effective with all three shRNA for RAB5C. Thus, we selected sh1 for RAB5A and B, and sh3 for RAB5C (since it induced the highest increase in exosome secretion as measured by the FACS based assay in **Figure 19A**) to analyze the results. Inhibition of RAB5A (sh1) induced a decrease of MHC class II and HSC70, although not of CD63, which mostly suggests a decrease of exosome secretion as we observed with the FACS-based assay. RAB5B invalidation (sh1) also seemed to result in a decrease in exosome secretion, albeit less important (in fact, the most effective decrease of exosome secretion was observed with sh3, which was the least effective in the FACS-based assay setting). Finally, RAB5C inactivation induced an exosome increase as evidenced by the increase in all markers considered.

We then studied exosomes secreted by RAB5A and C invalidated cells by immuno-EM to compare their morphology and CD63 and MHC class II content (**Figure 19C**). Vesicles secreted by shRAB5A-treated cells show a decrease of MHC class II in favor of a higher amount of unlabeled vesicles as compared to control vesicles, perhaps related to a difference in sorting. In shRAB5C conditions, we may observe an important increase of CD63+MHC II+ vesicles among the largest ones (> 100 nm).

Conclusion

These results collectively show that RAB5 is involved in the exosome secretion pathway, albeit with different effects according to the different isoforms. RAB5 proteins, and in particular some of its isoforms, are important for the maintenance of the homeostasis of early endosomes. We propose that the alteration of this homeostasis has consequences on the downstream endosome maturation, which may explain the modifications of exosome biogenesis and secretion that we observed.

DISCUSSION

DISCUSSION

Since their discovery three decades ago, exosomes have been extensively studied and many advances have been made to achieve their characterization. However, one important question has not been addressed so far, and that is the physiological relevance of exosomes *in vivo*. As mentioned before, a detailed knowledge of the molecular mechanisms involved in exosome biogenesis and secretion is required. I have thus focused my PhD studies on these mechanisms, and in particular, on three families of molecules (ESCRTs, Rabs, SNAREs) that, due to their known roles in protein sorting and incorporation into ILVs, intracellular trafficking, or the fusion of membranes from two different subcellular compartments, are potentially involved in the exosome secretion pathway.

The results from my studies have been presented in three parts:

1) Presence of different subpopulations of exosomes in 100,000 x *g* pellets: Rab27a-dependent and -independent vesicles

2) Function of ESCRT components in exosome biogenesis

3) Function of other molecules, especially SNARE proteins, in exosome biogenesis or secretion

In the following section, I will discuss these results in relation to the current literature, as well as the possible limitations of the study and potential perspectives to be explored.

1) Presence of different subpopulations of exosomes in 100,000 x *g* pellets: Rab27a-dependent and -independent vesicles

First, we performed a study on the different populations of vesicles observed in mouse mammary carcinoma 4T1 cells. Experiments were achieved on control and Rab27a-invalidated cells, and included several approaches to characterize the secreted vesicles, namely western blotting, electron microscopy, and floatation in overlaid sucrose gradients. This in-depth study of extracellular vesicles showed that the protocol most widely used to purify exosomes by differential centrifugation was unable to separate two populations of vesicles, which displayed differences in terms of size, density, enrichment in selected proteins, and a different requirement of Rab27a for their secretion. These different characteristics point to diverse intracellular origins, thus suggesting that one of the populations may not correspond to endosome-derived exosomes but rather to vesicles of a different origin, perhaps the plasma membrane. This study also challenged the perception of two commonly used "markers" of exosomes as specific proteins only found in these vesicles, since their presence was also detected in larger microvesicles.

1.1 Heterogeneity of exosomes

The heterogeneity of exosomes was previously observed mostly in terms of size: most studies describe purified vesicles within a range of sizes between 30 and 100 nm. This heterogeneity in size has been demonstrated by electron microscopy in many cell types, including EBV-transformed B cells (Raposo et al., 1996), dendritic cells (Andre et al., 2004; Segura et al., 2005; Zitvogel et al., 1998), T cells (Blanchard et al., 2002), tumor cell lines (Wolfers et al., 2001), malignant effusions (Andre et al., 2002), primary cortical neurons (Faure et al., 2006), basophils (Laulagnier et al., 2004a), intestinal epithelial cells (Mallegol et al., 2007; van Niel et al., 2001), HeLa-CIITA cells (Ostrowski et al., 2010), etc.

Heterogeneity in the protein content of exosomes has been observed by immuno-EM in several cell types, through the detection of several proteins. For instance, it was evidenced in human (Andre et al., 2004) or mouse (Zitvogel et al., 1998) DCs with markers MHC class I, II and tetraspanins CD81 or CD63; in melanoma and ovarian cancer (Andre et al., 2002) with markers MHC class I, II, and melanoma-associated antigens TRP1 and gp100 (PMEL); in Jurkat T cells (Blanchard et al., 2002) by detecting TCR-beta; in EBV-B cells (Escola et al.,

1998; Raposo et al., 1996) with markers CD82, CD53, and MHC class II; in prion protein infected rov cells (Fevrier et al., 2004) with PrP, flotillin, and Tsg101; and in intestinal epithelial cells (Mallegol et al., 2007) by detecting CD9, CD81, CD82, and A33 antigen. Micrographs show the presence of vesicles not labeled for a particular protein, and labeled vesicles with varying levels of staining, indicating different levels of the molecule in individual particles. In most studies these differences in protein content between vesicles of the same preparation were not analyzed quantitatively. One study, however, focusing on the description of exosomes secreted by the apical and basolateral sides of intestinal epithelial cells (van Niel et al., 2001), did quantify the proportion of vesicles labeled for the markers CD63 and MHC class II or CD26 and MHC class II, and this analysis indicated qualitative differences between the two types of exosomes: apical exosomes were mostly unlabeled, while basolateral exosomes showed a higher proportion of vesicles labeled with CD63, MHC class II, or labeled with both antigens, as compared to apical exosomes. Heterogeneity in protein content was also shown by floatation on sucrose gradients in one report performed on EBV-B cell- derived exosomes purified by ultracentrifugation at 70,000 x *g* (Escola et al., 1998), in which CD82 was mostly enriched in less dense vesicles (1.23 g/mL), whereas the highest levels of MHC class II and CD81 were detected on vesicles with a higher density (around 1.35 g/mL). **Table 3** summarizes the results of these different articles in terms of variability in size and protein content. Yet in most reports several markers are not analyzed simultaneously. Generally, the visualization of one single protein is used as evidence of the identity of exosomes, and detected in the same fraction(s) as a protein considered as marker of exosomes. For example, Acyl-TyA was observed to co-fractionate with CD63 in Jurkat cells (Fang et al., 2007); an overexpressed cytosolic YFP with ALIX and TSG101 in neurons (Faure et al., 2006) and PLP with Alix in an oligodendroglial cell line (Trajkovic et al., 2008).

Our results extend these findings by characterizing in more detail vesicles secreted by a mouse cell line, through the combination of western blotting and separation by sucrose gradients followed by a quantification of vesicles analyzed by immuno-EM, which is rarely performed. This allowed us to detect a population of vesicles that didn't require Rab27a for their secretion. These vesicles did not harbor markers of the endosomal compartments (CD63, Alix, Tsg101) but presented cytosolic or plasma membrane markers (CD9 and Mfge8), strongly suggesting that these vesicles may, in fact, not correspond to endosome-

Reference	Cell type	Exosome size (nm)	Determination of exosome heterogeneous protein content by immuno-electron microscopy (IEM) or WB of density gradient fractions (WB)	Purification technique
Raposo et al, 1996	EBV-B cells (human)	60-80	MHC II (IEM)	Centrifugation at 70,000 x g
Zitvogel et al, 1998	DCs (human)	50-90	CD63, MHC I, MHC II (IEM)	Centrifugation at 100,000 x g
Escola et al, 1998	EBV-B cells (human)	n.d.	CD82, CD53, MHC II (IEM); CD81, CD82, MHC II (WB of sucrose gradient fractions)	Centrifugation at 70,000 x g, sucrose gradient
van Niel et al, 2001	Intestinal cell lines (human)	30-90	CD63, CD26, MHC II (IEM)	Centrifugation at 100,000 x g
Wolfers et al, 2001	Mastocytoma cell line (P815, mouse)	40-100	n.d.	Centrifugation at 100,000 x g
Blanchard et al, 2002	Jurkat T cell line (human)	50-100	CD3, TCR β (IEM)	Centrifugation at 100,000 x g
Andre et al, 2002	Melanoma or ovarian cancer (human)	50-100	CD81, MHC I, MHC II, TRP1, gp100 (IEM)	Centrifugation at 100,000 x g, sucrose/D_2O cushion
Fevrier et al, 2004	Prion infected Rov cells (rabbit)	50-90	PrP, flotillin, Tsg101 (IEM)	Centrifugation at 100,000 x g, sucrose gradient
Laulagnier et al, 2004	Basophil cell line (rat)	60-90	n.d.	Centrifugation at 110,000 x g
Andre et al, 2004	Monocyte-derived DCs (human)	60-90	CD81, MHC I, MHC II (IEM)	Centrifugation at 100,000 x g, sucrose/D_2O cushion
Segura et al, 2005	D1 DC cell line, BMDCs (mouse)	50-100	n.d.	Centrifugation at 100,000 x g
Faure et al, 2006	Cortical neurons (rat)	Average 100 nm	n.d.	Centrifugation at 100,000 x g, sucrose gradient
Mallegol et al, 2007	Intestinal cell lines (human)	30-90	CD9, CD81, CD82, A33 antigen (IEM)	Centrifugation at 100,000 x g
Ostrowski et al, 2010	HeLa cell line (human)	50-100	n.d.	Centrifugation at 100,000 x g

TABLE 3 - Summary of studies showing the heterogeneity of exosomes.
In this table are listed some studies which show the heterogeneity of purified exosomes in terms of size, and protein content. Size ranges were measured based on electron microscopy (EM) observation, and protein content was determined by immuno-EM observation (IEM) of the indicated markers, or western blotting (WB) analysis of exosome fractions obtained by density gradient separation. Purification techniques are detailed in the last column. n.d. not determined

derived exosomes, but are likely to be plasma membrane derived microvesicles. These cells, similarly to tumor cells that are clearly known to also secrete microvesicles, thus secrete different types of vesicles that are all isolated with the same method. It is not clear if different cell types are also capable of secreting different populations of vesicles, and this should therefore be analyzed in cells from different origins.

1.2 Purification techniques and exosome subpopulations

The identification of different populations of vesicles in a single preparation is hindered by the currently available purification and detection techniques. Most purification methods only enrich a preparation in a certain type of vesicle, without actually achieving a pure sample. Sucrose gradients can improve this purification, but as of yet they have not been shown to be able to separate vesicles of different sizes under 100 nm. Likewise, most detection techniques do not distinguish individual vesicles, and are instead useful to characterize bulk populations of secreted vesicles. We must therefore rely on electron microscopy, which is a powerful although less readily available technique, and time-consuming in terms of quantification purposes. Furthermore, few proteins may be analyzed simultaneously in a sample, and not all antibodies may be employed with this technique due to the fixation steps that are performed.

Hence, the difficulty in working with exosomes lies in the limited techniques we dispose of today. Novel purification methods allowing the separation of vesicles with sizes similar to exosomes would enable us to better understand the characteristics of subpopulations of exosomes, in terms of size, density, molecular composition, and possibly in terms of variable effects on target cells. This might also contribute to our understanding of other secreted vesicles, and thus provide a more comprehensive depiction of the different vesicles that may be secreted by a single cell type.

A recent article reported the comparison of three different methods of exosome purification: ultracentrifugation, density gradient centrifugation (Optiprep gradient), and immunoaffinity capture (using beads coated with antibodies to EpCAM, an epithelial cell adhesion molecule, which was shown to be overexpressed in epithelial carcinomas) (Tauro et al., 2012). Exosomes secreted by a colon carcinoma cell line were purified following the different protocols, analyzed by western blotting (including the markers ALIX, HSP70 and TSG101) and EM, and quantitative proteomics were then performed. This analysis shows

the variable protein composition of exosomes purified by the different methods, thus supporting the concept of heterogeneous populations of exosomes. Furthermore, the authors conclude that the best purification method was the immunoaffinity capture, since less contaminating vesicles were seen by EM (as opposed to the ultracentrifugation technique), and the preparations were more enriched in exosome-associated proteins than with the other two methods. However, an immuno-EM analysis of the secreted vesicles with an EpCAM labeling would have been useful to assess what proportion of the vesicles were likely to be trapped on the beads.

1.3 Exosomal protein enrichment and exosomal markers

Another important question raised by these experiments concerns the so-called exosomal "markers". The presence of several proteins has been detected in exosomes by numerous proteomic studies, and many of them have been described as enriched in exosomes as compared to total cell lysates by western blotting. However, referring to a certain protein as a "marker" of exosomes implies that said molecule is only found on these vesicles, and can thus be used reliably to detect exosomes and no other contaminating vesicle. As shown here, two proteins that have been extensively used as markers of exosomes (CD9 and Mfge8) can also be identified in microvesicles, and should therefore not be considered as markers exclusive of exosomes. Since they are enriched in exosomes, they may still be employed as a means to characterize exosomes, but only in combination with several other markers in order to ensure that the correct vesicle is being identified. An observation was made recently upon analysis of human prostasomes (i.e. exosomes secreted by prostate epithelial cells and found in the seminal fluid) (Aalberts et al., 2012). In their study the authors describe two populations of vesicles, with different sizes and protein composition (smaller vesicles containing the protein GLIPR2 in their lumen, and larger vesicles containing annexin A1 at the surface of the vesicles), but showing the presence of CD9 and a similar equilibrium density after 62 hours of ultracentrifugation (1.15 g/mL). When the vesicles were spun in density gradients for 16 hours, the two populations with different densities were observed: one with densities around 1.09-1.16 g/mL corresponding to the larger vesicles and the smaller vesicles which floated at 1.23-1.26 g/mL. This observation is consistent with our findings, since we also found the presence of two subpopulations of vesicles by performing the analysis of protein contents of exosomes separated by sucrose

gradients, and observed an enrichment of CD9 in larger vesicles (1.11-1.14 g/mL), as well as in smaller ones (1.26-1.29 g/mL). It would be interesting to assess whether in our model GLIPR2 is enriched in high density fractions, and annexin A1 in low density fractions, as observed with prostasomes. The authors also raise the question of inter-individual variability in the protein composition of exosomes: they discovered that the vesicles purified from samples from different donors did not always contain the prostate-specific protein PSCA (prostate stem-cell antigen), thus evidencing differences in exosome composition between individuals. This aspect of exosome biology has not been extensively studied, and efforts in this direction should be made since this could account for the variability between exosomes released by different cell lines.

The possibility remains that many of the proteins used today as markers of exosomes are equally not specific of this type of vesicles. We should also keep in mind that the presence of many of these proteins on exosomes may be specific of the cell type that secretes them. A clear knowledge of the distribution of these different markers at the cellular level for a given cell type is an essential complement when analyzing exosomes. Very few reports have focused simultaneously on vesicles from different intracellular origins, or of different sizes (Heijnen et al., 1999; Thery et al., 2001). Efforts should therefore be made in comparing exosomes and other vesicles such as microvesicles, to determine which markers are specific of which type of vesicle, and which are found in both. Proteomic studies of both types of vesicles (for instance, purified by ultracentrifugation at 10,000 x g for microvesicles and 100,000 x g for exosomes) would probably provide an interesting starting point, and since this is not a quantitative technique it should be followed by a western blotting analysis of selected candidates performed on equal amounts of total proteins from each type of vesicles, to assess both the presence and enrichment of these candidate molecules in the different vesicles examined as compared to total cell lysates.

2) Function of ESCRT components in exosome biogenesis

The second focus of my studies aimed at analyzing the potential role of ESCRT proteins in exosome biogenesis. ESCRT proteins are known to be involved in MVE formation in yeast, and this has also been observed in mammalian cells, but more recently, reports have emerged that challenge the consideration of the ESCRT pathway as the only mechanism responsible for this process (Simons and Raposo, 2009). An RNA interference screen indicated that seven ESCRT components affected the exosome secretion pathway: exosome secretion was reduced in cells invalidated for HRS, STAM1 and TSG101, and it was increased in cells with CHMP4C, VPS4B, VTA1 and ALIX inhibition. This reinforces the notion that ESCRTs are necessary for ILV biogenesis. The invalidation of the three subunits of the ESCRT-II complex SNF8/VPS22, VPS36 and VPS25 has evidenced no significant alterations of the secretion of exosomes as measured by our FACS-based assay. This observation is consistent with reports that ESCRT-II subunits are seemingly not involved in HIV budding (Langelier et al., 2006), or in the degradation of the EGFR (Bowers et al., 2006), two processes known to require selected ESCRT proteins. A single ESCRT-III component, CHMP4C/VPS32C was shown to modulate exosome secretion, whereas the other components were either unable to consistently modify exosome secretion (CHMP1B, CHMP5, CHMP6/VPS20), or we were unable to further analyze the results due to the inability to demonstrate the downregulation of the targeted gene (CHMP2A, CHMP2B, CHMP4A/VPS32A, CHMP4B/VPS32B, VPS24). We can therefore not rule out the possible involvement of some of these components, and we must keep in mind that the existence of several isoforms of the four subunits of ESCRT-III implies that there might be a redundancy of these proteins in mammalian cells.

In contrast, early proteins of the ESCRT pathway such as HRS, STAM1 (both ESCRT-0) and TSG101 (ESCRT-I), as well as late components such as VPS4B, VTA1 and ALIX, induced a significant modulation of exosome secretion upon invalidation of the gene expression. A validation step involving the purification of exosomes by differential ultracentrifugation, and the analysis of three exosomal markers by western blotting, showed similar results for most of the candidates: vesicles released by HRS, STAM1 or TSG101-invalidated cells evidenced a decrease in total yield, and VPS4B inhibition caused an increase in exosome secretion. These results are consistent with several studies showing the requirement of these molecules for the formation of MVEs. VPS4 was shown to be involved in the release of ESCRT-III proteins

from the endosome limiting membrane, which is necessary for the correct morphology of these compartments (Babst et al., 1998). Hrs inactivation induced the presence of enlarged endosomes and a decrease in the number of MVEs, as well as a decrease in the recruitment of ESCRT-I components (Bache et al., 2003). Downregulation of TSG101 inhibited the formation of MVEs (Razi and Futter, 2006). CHMP4C and VTA1 showed conflicting results with the screening and were therefore not further analyzed. One possible way of explaining this discrepancy would be to consider that control (SCR) preparations have less vesicles bearing both CD63 and MHC class II molecules than shVTA1 or shCHMP4C preparations (thus resulting in a lower detection of exosomes with the FACS-based assay in control conditions), but proportionally more vesicles with either CD63 or MHC class II, thus increasing the signal in western blotting experiments for the control exosomes as compared to the other two conditions. The invalidation of ALIX evidenced an interesting effect: whereas the FACS based screen had showed an increase in the release of CD63/MHC class II/ CD81-positive vesicles, western blotting experiments performed on purified exosomes showed a similar level for two of the markers (CD63 and HSC70), and a marked increase in MHC class II content in these vesicles. This observation implies that the increase observed in the FACS based assay conditions is in fact not an increase of total exosome secretion, but an increase in MHC class II content of the vesicles. The results of the FACS-based assay and the validation of shALIX-derived vesicles are, however, consistent, since the detection of CD63-positive vesicles trapped onto beads is done with anti-CD81 and anti-MHC class II antibodies.

The increase in exosome secretion observed upon inactivation of VPS4B was quite unexpected. The correct function of the AAA-ATPase is essential for the recycling of ESCRT-III components VPS24 and CHMP4 in particular (Babst et al., 1998), and constitutes the only energy consuming member of the ESCRT family, which allows this molecule and its cofactor VTA1 to separate ESCRT proteins from the limiting membrane of endosomes (Sachse et al., 2004). Thus, the accumulation of ESCRT components on the endosomal membrane caused by the inactivation of VPS4 should entail a complete halt of the ESCRT machinery altogether, and thus prevent the formation of ILVs. The increase in exosome secretion, and the presence of MVEs as observed by electron microscopy (data not shown) in VPS4B-invalidated cells suggests that mechanisms other than the ESCRT pathway might coexist in cells and be responsible for the formation of MVEs. A report showing the presence of MVEs

in cells depleted for all four ESCRT complexes simultaneously (Stuffers et al., 2009) is consistent with this possibility: cells inactivated for Hrs, Tsg101, Vps22 and Vps24 evidenced an alteration of the morphology of endosomes, and the presence of MVEs containing enlarged ILVs into which EGFR molecules are not sorted. It is thus tempting to speculate that upon blocking of the ESCRT pathway, another mechanism might subsequently be increased to compensate for the lack of ESCRT function, and would thus generate the observed increase in exosome yield. Two other mechanisms of ILV formation have been described in different cell types, namely ceramide (dependent on the activity of the enzyme neutral sphyngomyelinase) in oligodendroglial cells (Trajkovic et al., 2008), and the tetraspanin CD63 in melanocytes (van Niel et al., 2011). Thus, it might be of interest to simultaneously block the ESCRT pathway in combination with another mechanism (for instance, through the invalidation of CD63), to observe if MVE biogenesis is (almost) completely blocked. Preliminary experiments were performed for this purpose by co-infecting cells with lentiviruses bearing shALIX or shVPS4B, and lentiviruses with an shRNA directed against CD63, simultaneously. However, since both viral particles carried the same resistance gene (puromycin), we were unable to select cells that had been infected with both viruses, and instead relied on a bulk population analysis of the downregulation of both proteins by western blotting. A way to avoid this problem would be to employ lentiviruses with different resistance genes, or lentiviruses that carried sequences for fluorescent protein expression, which would allow us to select infected cells by FACS sorting, as well as by antibiotic treatment.

2.1 Exosome subpopulations secreted by HeLa-CIITA cells

Immuno-EM observation of CD63 and MHC class II labeled vesicles was performed for each condition. As observed for 4T1 mouse tumor cells, the 100,000 x *g* pellet obtained from control HeLa-CIITA cells also showed the presence of different populations of vesicles by quantitative immuno-EM: in particular, one smaller population (30-60 nm diameter) was enriched in CD63, whereas larger vesicles (> 100 nm diameter) were enriched in MHC class II molecules. The existence of different populations of exosomes among secreted vesicles is in agreement with previous reports showing the presence of different MVEs in a single cell (Buschow et al., 2009; Mobius et al., 2003; White et al., 2006), as these different exosomes may be formed in the diverse MVEs. Alternatively, larger vesicles might potentially

correspond to shedding microvesicles, due to their larger size between 100 and 1000 nm as described in activated platelets (Heijnen et al., 1999), and since MHC class II is also located at the plasma membrane of cells, unlike CD63 which is mostly found on the limiting membrane of late endosomes and lysosomes, and less present at the plasma membrane. One way of evaluating this might be to infect these cells with shRAB27A-bearing lentiviruses, and after obtaining the purified vesicles secreted by the invalidated cells, performing a similar immuno-EM staining: if larger vesicles are shed directly from the plasma membrane it is conceivable that their secretion would not be affected by the invalidation of RAB27A, whereas the release of smaller vesicles might be reduced. In the mouse mammary carcinoma model 4T1, we observed the presence of smaller vesicles enriched in CD9 and Mfge8 whose secretion was independent of Rab27a function, and we hypothesized that these vesicles corresponded to microvesicles shed from the plasma membrane. These observations raise another question concerning the differences between vesicles secreted by different cell types: could vesicles being shed from the plasma membrane be of different sizes according to which cell type secretes them? This could be addressed by inhibiting exosome release through the invalidation of Rab27a in different cell types, collecting vesicles centrifuged at 10,000 and 100,000 x g, and performing a similar study as the one described with the 4T1 model.

The presence of MHC class II on vesicles other than CD63-enriched exosomes is similar to results obtained in a rat basophilic cell line, RBL (Laulagnier et al., 2005), where cells were labeled with fluorescent lipids, and subsequently the presence of these lipids was analyzed in exosomes with a different protein composition by trapping the vesicles on antibody-coated beads against MHC class II, CD63 or CD81. FACS analysis of the exosomes trapped on beads showed that MHC class II positive exosomes were enriched in Bodipy-phosphocholine, whereas exosomes positive for CD63 or CD81 were labeled with both Bodipy-Ceramide and Bodipy-phosphocholine. These observations suggested the existence of two subpopulations of vesicles with different protein and lipid compositions. However, these experiments were performed by trapping exosomes on beads and measuring fluorescent lipids by FACS, thus they did not provide any information on the size of these vesicles, and a further description of other markers that may help to differentiate these different populations was not performed. It might also be of interest in our models to investigate the lipid content of the different vesicle populations, by separating vesicles of

low density (around 1.13-1.15 g/mL) and those of higher density (around 1.26-1.29 g/mL), extracting lipids and measuring the different lipid species present as described elsewhere (Laulagnier et al., 2004b; Wubbolts et al., 2003), to see if there are differences between the different subtypes of vesicles.

2.2 ESCRT proteins and exosome subpopulations

The evaluation of the size and protein content of individual vesicles allowed us to uncover differences in the nature and composition of the vesicles upon invalidation of the different candidate ESCRT proteins. This probably reflects the specific role that each ESCRT protein plays in the maturation of endosomes. The invalidation of HRS, STAM1, and TSG101 induced a decrease in the total amount of secreted vesicles, but the nature of the remaining secreted vesicles was slightly different in the different conditions: in particular, the size of the vesicles was variable. This is in agreement with the observation that the invalidation of each protein in human cell lines results in different effects on endosome maturation (Razi and Futter, 2006): TSG101 depletion inhibits MVE formation and causes the vacuolar portion of the early endosome to tubulate, whereas HRS depletion does not result in the tubulation of early endosomes, but induces the formation of enlarged MVEs that are able to fuse with lysosomes. EM observation of the shRNA-treated HeLa-CIITA cells would have been worthwhile, so as to assess if the MVEs of TSG101 and HRS-invalidated cells behave similarly to what was observed in the aforementioned article. This technique would also allow us to determine if MVEs possess smaller or larger ILVs in HRS or STAM1-depleted cells, respectively.

In light of the results discussed above regarding the specificity of the markers used to identify exosomes, it would be interesting to perform western blots with vesicles pelleted at 10,000 x g, as a means to see if the proteins measured in this study to define exosomes are specific of these vesicles, or if some of them can also be found on vesicles from other intracellular origins. In the 4T1 model, we observed the presence of CD9 in microvesicles pelleted at 10,000 x g, but we were unable to detect CD63 in these vesicles by western blotting: CD63 thus appeared as a marker specific of exosomes, unlike CD9. CD63, Tsg101, Alix and Hsc70 were found to be good markers of Rab27a-dependent exosomes. In the HeLa-CIITA model, anti-CD63 antibodies are available for immuno-EM analysis, and the quantification of CD63 staining performed on the 100,000 x g pellet showed that CD63 is

mostly enriched in smaller vesicles, between 30-60 nm diameter. In order to determine if CD63 is a good exosomal marker, it would be important to determine if these smaller vesicles are in fact generated as ILVs in MVEs, or if they are shed directly from the plasma membrane. The analysis of immuno-EM images of MVEs to search for the presence of these vesicles did not allow the observation of many smaller vesicles (either due to uranyl acetate contrast issues, or perhaps due to the fact that ILVs are packed together in MVEs, thus preventing the observation of smaller vesicles), making it therefore difficult to conclude if the CD63-enriched small vesicles correspond to ILVs. On the other hand, the observation of the plasma membrane by immuno-EM did not evidence the presence of CD63, but mostly that of MHC class II (which was also highly enriched in larger vesicles), thus suggesting that these vesicles are not originated in the plasma membrane. Floatation of the 100,000 x *g* pellet into overlaid sucrose gradients, followed by western blotting to detect at least these three markers (MHC class II, CD63 and HSC70) might also give information as to subtle differences in density between vesicles from different fractions associated with the enrichment in a particular marker.

2.3 Effect of ALIX invalidation on MHC class II levels and sorting

The invalidation of ALIX in HeLa-CIITA cells caused an increase in MHC class II content in the 100,000 x *g* pellet as evidenced by western blotting. Immuno-EM analysis of the secreted vesicles also showed an increase in the size of the vesicles, as well as a general increase in MHC class II in vesicles of all sizes. These observations thus suggest that ALIX might be involved in the sorting of MHC class II onto the ILVs. A previous study has shown that ALIX interacts with the TfR through a specific cytoplasmic domain on the receptor, and can thus induce the sorting of the TfR into ILVs during the maturation of reticulocytes (Geminard et al., 2004). It would therefore be interesting to evaluate if MHC class II molecules somehow interact with ALIX, and if this interaction is required for the sorting of MHC class II into exosomes. In a different cell type, dendritic cells, it has been shown that the ubiquitination status of MHC class II molecules is essential for their trafficking to the degradative pathway or to the exosome secretion pathway (Buschow et al., 2009). In immature dendritic cells, ubiquitination of MHC class II molecules targets them to lysosomal degradation, whereas upon cognate interactions with antigen-specific T cells, ubiquitination of MHC class II is abrogated, and MHC class II molecules are sorted to the exosome secretion pathway

through a mechanism dependent on CD9-containing detergent resistant membranes. It would thus be important to investigate whether MHC class II molecules are ubiquitinated in the HeLa-CIITA model, and how this affects the trafficking of these molecules to the degradative or secretory pathway.

The analysis of both HeLa CIITA and human primary dendritic cells invalidated for ALIX has shown a significant increase of MHC class II molecules, both at the cell surface as well as in intracellular compartments. This observation suggests that ALIX may participate in the intracellular trafficking and degradation of these molecules. Given that it has recently been reported that an increase of MHC class II levels occurs upon CD63 silencing (Hoorn et al., 2012), and the findings that ALIX interacts with syntenin (Baietti et al., 2012), which in turn interacts with CD63 (Latysheva et al., 2006), it is possible that ALIX is involved in a CD63-dependent MHC class II degradation in both cell types. Further studies are required to demonstrate this, possibly by impairing the interaction between the different molecules that were just mentioned.

Interestingly, the analysis of HeLa cells treated with an siRNA against ALIX showed a significant reduction of exosome secretion by these cells, as shown by the significant decrease of CD63, syndecan and syntenin, and a reduction of HSP70 by western blotting (Baietti et al., 2012). In our study, shRNA-induced depletion of ALIX induced a significant increase in MHC class II molecules in the secreted vesicles, and a decrease in the proportion of vesicles not bearing MHC class II molecules, including CD63-positive vesicles (as shown by immuno-EM quantifications). By western blotting, we observed a slight decrease of CD63 and HSC70, although not a significant one, and an increase in MHC class II, which suggested an alteration in the molecular composition of the exosomes more than an actual increase in exosome secretion. It therefore seems that analyzing a different set of exosomal proteins by western blotting may lead to analyzing different vesicles altogether. An immuno-EM analysis where vesicles are quantified individually is therefore useful so as to obtain data not only on the vesicles that possess a particular protein marker, but also on the rest of the vesicles that may be negative for said marker, which are overlooked by a western blotting analysis.

A limitation of this study lies in the fact that we only employed one shRNA sequence to perform the experiments after the screening. It would thus be of interest to attempt complementation experiments using an shRNA-resistant version of the ALIX gene, to

evaluate if the restoration of endogenous levels of ALIX induces a similar phenotype as observed in control cells.

3) Function of additional molecules in exosome biogenesis or secretion

3.1 SNAREs

In the third part of my studies, we sought to investigate the implication of a panel of SNARE molecules in the secretion of exosomes by HeLa-CIITA cells. Due to their role in membrane fusion between different intracellular compartments, SNARE proteins are potential candidates to constitute the machinery involved in the final steps of exosome secretion, whereby the MVEs fuse with the plasma membrane and release the ILVs as exosomes into the extracellular space. Several genes of the 21 we analyzed did not show any modulation of exosome secretion upon invalidation, or the results obtained were not consistent between the different shRNA sequences targeting the different genes. However, some candidates evidenced a significant increase (YKT6, STX1, GOSR1) or decrease (STX2, SEC22L3) in exosome secretion when downregulated. These molecules might therefore be a part of the SNARE complex involved in the fusion of the MVE limiting membrane to the plasma membrane.

As mentioned before, few studies have addressed the requirement for SNAREs in the secretion of exosomes. Our results are inconsistent with the finding that VAMP7 is involved in exosome release by K562 cells (Fader et al., 2009): expression of the N-Terminal domain of VAMP7 which impairs the formation of the SNARE complex induced the accumulation of enlarged MVEs at the cell periphery, and reduced exosome secretion as measured by AChE enzymatic activity. There is, however, one report showing that VAMP7 is not required for the secretion of exosomes by MDCK cells (Proux-Gillardeaux et al., 2007), and would thus be in agreement with the findings we obtained: in this article, expression of the Longin domain (N-Terminus) of VAMP7 inhibits the secretion of lysosomes, but not that of exosomes as assessed by EM observation and western blotting with markers HSP70 and calreticulin. VAMP7 would be rather involved in the exocytosis of lysosomes as part of the complex, which includes also STX4 and synaptotagmin 7 (Rao et al., 2004). The findings of these

articles seem to indicate that in different cell types, different SNARE complexes might be involved in the fusion of certain compartments, as suggested by the conflicting results obtained with regard to the implication of VAMP7 in exosome secretion. It is also possible that different SNARE complexes could mediate the fusion of different subpopulations of MVEs within a single cell type, and thus the downregulation of different SNARE proteins might only affect the secretion of a particular subpopulation of exosomes. The quantification of several markers and of exosome size by immuno-EM in cells invalidated for selected SNARE elements compared to control cells, as performed in our previous articles, might provide insights into the latter possibility.

It should be stressed, however, that in this study only 21 candidates were analyzed, and 36 SNARE proteins have been described in human cells (Jahn and Scheller, 2006). Also, the qRT-PCR to measure the downregulation of each gene in the shRNA-treated cells has not been yet performed, and we can therefore not convincingly conclude on the role of the genes analyzed here in the secretion of exosomes as of yet.

The fact that three candidate proteins were able to increase exosome yield when invalidated (YKT6, STX1, GOSR1) is intriguing: if we suppose that the downregulation of a member of the SNARE complex will inhibit the formation of said SNARE complex, then by targeting members of the SNARE complex involved in the exosome pathway we should inhibit the fusion of MVEs with the plasma membrane and thus inhibit exosome secretion, not increase it. One possible explanation for these observations could be based on the fact that different SNARE complexes are involved in the secretion of exosomes formed in different MVEs within a single cell type. We could imagine that CD63/MHC II/CD81-positive vesicles are formed in a certain type of MVE, whose secretion is not inhibited by the targeting of these three proteins, and thus to compensate for the decrease of secretion of exosomes from other MVEs, the secretion of MVEs containing triple positive vesicles would be increased. This should be evaluated by western blotting and EM experiments of the obtained vesicles, to see if triple positive vesicles are indeed increased whereas other vesicles (unlabeled, or single positive) are decreased.

3.2 Other molecules

The implication of additional molecules that had previously been proposed in the literature as potentially involved in either the biogenesis or the secretion of exosomes was also

investigated. For this purpose, we exploited the screening that we developed in HeLa CIITA cells. We observed that several previously proposed candidates either had no effect on the amount of secreted exosomes as compared to control cells (PLD2, ANXA2, ATP6V0a4), or the effects were inconclusive due to inconsistencies between the different shRNA sequences (DGKA, BIG2, CIT).

The inactivation of some genes did impact on exosome secretion: the invalidation of SMPD3, ATP6V0a1and ATP6V0a3 resulted in an increase in exosome secretion, whereas that of TSAP6 and ATP6V0a2 induced a decrease. These last two results are consistent with previous reports. TSAP6 inhibition was shown to decrease exosome secretion in mammalian cells (Amzallag et al., 2004; Lespagnol et al., 2008; Yu et al., 2006). The mutation of the v-ATPase in the worm *C. elegans* showed an accumulation of small MVEs and defects in cuticle formation, suggesting their inability to fuse with the membrane (Liegeois et al., 2006). In the case of the v-ATPase, the results are difficult to interpret, since not all subunits behave similarly. This could point to different mechanisms operating in different cells, and also to the possibility that some subunits of the v-ATPase are critical in the release of exosomes whereas others are not within a given cell type.

Finally, and in contrast to the results obtained on the mouse oligodendroglial cell line (Trajkovic et al., 2008) where chemical inhibition or genetic downregulation of the type II neutral sphingomyelinase induced a decrease in the secretion of PLP-bearing exosomes, we observed an increased exosome secretion upon inactivation of the neutral sphingomyelinase II (SMPD3). We treated HeLa-CIITA cells with the inhibitor of neutral sphingomyelinases GW4869, and measured exosome secretion with the use of the FACS-based assay: we observed a decrease in exosome secretion by cells treated with the inhibitor (data not shown). The discrepancy between the results obtained with the shRNA and the inhibitor treatment might be due to the fact that at least two genes, *SMPD2* and *SMPD3*, encode for neutral sphingomyelinases (another gene *SMPD4* has been identified, but shares no homology with the other two), and thus the inhibitor might target SMPD2 as opposed to an SMPD3 decrease induced by the shRNA. Studies performed in a melanocytic cell line, also indicate that production of ceramide is not required for the sorting of CD63 or the melanosomal protein PMEL into ILVs, as shown by the use of inhibitors of the enzymatic activity, or by means of siRNA against type II sphingomyelinase (van Niel et al., 2011). Thus, the results obtained in the former study might only be applicable to this specific cell line,

and not a widely used mechanism for protein sorting and ILV formation in most cells types. It should also be noted that the former study followed the fate of exosomes bearing the protein PLP, and we analyzed the secretion of exosomes bearing CD63, MHC class II and CD81 simultaneously. The results might therefore not be comparable if the subpopulations of vesicles that were analyzed in both cases are different, since as discussed before different populations of exosomes may arise from different mechanisms of biogenesis of the MVEs. In this case also, a qRT-PCR was not performed for the moment, to monitor the correct downregulation of the targeted genes. Therefore, these experiments should be performed before drawing definite conclusions.

3.3 RAB5 isoforms

Finally, the screening previously described was once again useful to detect differences in the level of exosomes secreted by cells where the expression of different isoforms of the small GTPase Rab5 was abolished. The invalidation of RAB5A and RAB5B induced a decrease in exosome secretion (observed with 2 of the 3 shRNA sequences used to downregulate each gene), whereas RAB5C inhibition resulted in an increase. These results are consistent with unpublished results from our group obtained during the screening of Rab proteins to discover their function in exosome secretion (Ostrowski et al., 2010). When this initial screen was performed, we observed a decrease in exosome secretion upon RAB5A invalidation (Ostrowski et al., 2010) and an increase upon RAB5C inhibition (unpublished observations). Studies on the role of the different RAB5 isoforms in intracellular trafficking showed that inhibition of RAB5C does not affect the degradation of the EGFR in HeLa cells, whereas the inhibition of RAB5A and RAB5B impairs the degradation of the EGFR and delays its trafficking from early to late endosomes (Chen et al., 2009). It is therefore possible that the retention in early endosomes of cargoes fated for degradation or for secretion in association with exosomes, induced by RAB5A and RAB5B inhibition, causes a decrease in the biogenesis of ILVs. Consequently to the inhibition of ILV formation there would be a decrease in exosome release by these cells. Exosomes purified from the supernatant of the different shRNA-treated cells by differential ultracentrifugation were then analyzed by western blotting. Total cell lysates were also processed by western blotting to measure the downregulation of each isoform: the level of each protein targeted by a specific shRNA was decreased, without the levels of the other two isoforms being altered (strengthening that

the shRNA sequences induced a specific downregulation of the targeted gene). The analysis of the protein content of exosomes was performed for the shRNA that induced the highest protein downregulation, and showed that the results were similar to those obtained with the FACS based assay, although the effect observed with RAB5B inhibition was much lower (i.e., the exosome secretion was less decreased as observed by western blotting than what was measured in the FACS-based assay). Nonetheless, it is important to bear in mind that only one western blotting experiment was performed, and thus additional experiments are required to finally demonstrate the involvement of the different isoforms in the biogenesis of exosomes.

These results, together with articles showing the involvement of different Rab proteins such as RAB27 (Ostrowski et al., 2010), RAB11 (Savina et al., 2005) and RAB35 (Hsu et al., 2010) in exosome secretion, confirm the relevance of this family of proteins in the exosome secretion pathway.

4) Perspectives and conclusions

The results obtained in this thesis illustrate the difficulties of working with exosomes. Their small size, the lack of a consistent definition of their characteristics and properties, the potential presence of other secreted vesicles in exosome preparations, and the existence of different subpopulations of vesicles within the exosome size range render the analysis of the results challenging. Breakthroughs in the field are further complicated by the lack of a consensus concerning the best protocols to both purify and analyze exosomes, and by the fact that most groups only focus on one type of secreted vesicle, without taking into account the potential presence of others in the purified material. This has consequences on the study of both the mechanisms of exosome biogenesis and secretion, as well as the possible functions that the different vesicles could have *in vitro* and *in vivo*. The activity of the newly created International Society of Extracellular Vesicles (ISEV) is thus essential to the coordination of future research in the field. In order to compare results obtained in different models or cell types, identical protocols must be used by the different research groups, which would allow the identification of common exosome markers as well as markers specific of the cell type evaluated. It is also important to systematically study other

types of secreted vesicles in parallel with exosomes (including cell lysates and cell debris), so as to assess the specificity of these markers. A combination of proteomic approaches, RNA sequencing, and identification of lipids in exosomes secreted by different cell types, as well as in different exosome subpopulations, would provide a more comprehensive view of the molecular composition of these different vesicles. These aspects are relevant when studying the function of exosomes: just to give an example, in terms of antigen-specific T cell activation, only exosomes enriched in MHC class II (or class I molecules) and co-stimulatory molecules will carry out this particular function, and as shown here only a fraction of the secreted vesicles is highly enriched in MHC class II.

In conclusion, despite the fact that we are at an early stage of understanding the biology of exosomes, as well as the mechanisms that control their biogenesis and secretion, the results presented in this manuscript indicate that cells secrete different subpopulations of exosomes and that the methods used to purify exosomes from different cells may not always yield exclusively endosome-derived exosomes. Thereby a more precise definition of the properties of exosomes, as well as means to differentiate them from other secreted vesicles, are lacking which hinders our understanding of the functions that these vesicles may accomplish. Therefore, novel purification methods shared by different laboratories should be developed. The study of the mechanisms involved in the exosome secretion pathway shows that exosome biogenesis and secretion is more complex then initially thought. Cells host different MVEs subpopulations, which are themselves heterogeneous in content. Several pathways may coexist in a single secreting cell and this may depend on its differentiation status, adding additional complexity to the field, but on the other hand helping to reconcile previous findings in the literature (i.e. ESCRT dependent or independent mechanisms for example). These aspects must be addressed in future investigations, in order to increase our current knowledge concerning exosomes, and provide tools to study the physiological relevance of this atypical secretion pathway.

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APPENDIX

APPENDIX

TABLE 4 - shRNA SEQUENCES

The sequences are listed from sh1 to sh5

A) SNARE PROTEINS

a) R-SNARE

VAMP3

TRCN0000029814	CGGGATTACTGTTCTGGTTAT
TRCN0000029815	GCAGCCAAGTTGAAGAGGAAA
TRCN0000029816	TGGTGGACATAATGCGAGTTA
TRCN0000029817	CAGGCGCTTCTCAATTTGAAA
TRCN0000029818	CGAGTTAACGTGGACAAGGTT

VAMP7 (SYBL1)

TRCN0000059888	GCGAGGAGAAAGATTGGAATT
TRCN0000059889	GCTCACTATTATCATCATCAT
TRCN0000059890	GAGCAGATTCTGGCTAAGATA
TRCN0000059891	GCACTTCCATATGCCATGAAT
TRCN0000059892	CGTACTCACATGGCAATTATT

YKT6

TRCN0000059763	GCCGAACTAGATGAGACCAAA
TRCN0000059764	CGCATACGATGTGTCTTCCTT
TRCN0000059765	GAGAAGCTGATCCCATGACTA
TRCN0000059766	CGGAATGATAGTCTTGCAGGT
TRCN0000059767	ACAGTCTAAAGCCTTCTATAA

b) Q-SNARE

STX1

TRCN0000065008	GAAGAACTCATGTCCGACATA
TRCN0000065009	CCAGAAAGTTTGTGGAGGTCA
TRCN0000065010	GAGGAGATTCGAGGCTTCATT
TRCN0000065011	AGGATCGAGTACAATGTGGAA
TRCN0000065012	CATAAAGAAGACAGCAAACAA

STX2 (EPIM)

TRCN0000065283	CGAGCCAAGTTAAAGGCTATT
TRCN0000065284	CCTAGCAACAACATTGTCCTA
TRCN0000065285	GCGTGTAGGAAGAATGATGAT

TRCN0000065286   CCATCTTCACTTCCGACATTA
TRCN0000065287   GCATGAGATGTTCATGGACAT

STX3A
TRCN0000065013   GCCCGGAAGAAATTGATAATT
TRCN0000065014   GCAGCTCACGACTGAGATTAA
TRCN0000065015   GCAGCTCGAAATTACTGGCAA
TRCN0000065016   GATTGAGGAAACTCGGCTTAA

STX4A
TRCN0000065023   CCGTCAACACAAGAATGAGAA
TRCN0000065024   CGGACAATTCGGCAGACTATT
TRCN0000065025   GCAATTCAATGCAGTCCGAAT
TRCN0000065026   CAGCAGCTTGAACGCAGTATT
TRCN0000065027   GCTGCACGACATATTCACTTT

STX5A
TRCN0000059823   CCAGACAAATAAGCCAGCTTT
TRCN0000059824   GCAGTCGAAACTGGCTTCTAT
TRCN0000059825   CCATTCAGAGATCCTCAAGTA
TRCN0000059826   CCTTAGCAACACATTTGCCAA
TRCN0000059827   GCAGAACATTGAGTCGACAAT

STX6
TRCN0000059463   GCAGGCATTAGCTGAAAGAAA
TRCN0000059464   GCATAGTTGAAGCAAATCCTA
TRCN0000059465   GCACTGGAACAACAGATAAAT
TRCN0000059466   GACAATGTGATGAAGAAACTT
TRCN0000059467   CACAGCAACAAGGGAAGAAAT

STX7
TRCN0000065053   CCAGAGGATCTCTTCTAACAT
TRCN0000065054   GCGATTATCAGTCTCATCATA
TRCN0000065055   CGTCTTATTCATGAGAGAGAA
TRCN0000065056   CAGCAGATTATCAGCGCAAAT
TRCN0000065057   GTTCACAACATCACTGACAAA
STX8
TRCN0000059828   GCCCAAGAAATTGCTGAGAAA
TRCN0000059829   CCTCTATCATAAGTCGCCAAA
TRCN0000059830   GCAGGAAATTGGGAATGAATT
TRCN0000059831   GCTGTGTCAACACATCAGATA
TRCN0000059832   CGTGACAATCAGAGCTTTGTT

STX12
TRCN0000065048   CGAGCTGCTTACTATCAGAAA
TRCN0000065049   CGCCAAGGAAACAAATGAATT
TRCN0000065050   GCAGGATTTGGAACTTATTAA

TRCN0000065051   GCCACTGCTCAGATAAAGAAT
TRCN0000065052   GCTTGTCCTGTCAGTGATTAT

STX17
TRCN0000060118   CCTGTCTTCTAACACCTCAAA
TRCN0000060119   CGATCCAATATCCGAGAAATT
TRCN0000060121   GCAGAATTTCTCCAACTCCAT
TRCN0000060122   CCAGCTATCCAGAAATTCATT

STX1A
TRCN0000065008   GAAGAACTCATGTCCGACATA
TRCN0000065009   CCAGAAAGTTTGTGGAGGTCA
TRCN0000065010   GAGGAGATTCGAGGCTTCATT
TRCN0000065011   AGGATCGAGTACAATGTGGAA
TRCN0000065012   CATAAAGAAGACAGCAAACAA

STX1B
TRCN0000065263   GCACGATATGTTTGTGGACAT
TRCN0000065264   CGTGGAGGTAATGACCGAATA
TRCN0000065265   GCGAAAGACAGTGATGATGAA
TRCN0000065266   ACACCAAGAAAGCAGTGAAAT
TRCN0000065267   GCCAACAAGGTTCGGTCCAAA

GOSR1
TRCN0000060383   GCGACAGGTATAGTTCTGATA
TRCN0000060384   GCAGGATTATACACATGAATT
TRCN0000060385   CGAAACTCAGATCGTCTGATA
TRCN0000060386   CGATTGAGATTGAACAACTTT
TRCN0000060387   CAAAGCAAACTTTATGGCAAT

GOSR2
TRCN0000060488   CCACCATACCAATGGACGAAT
TRCN0000060489   CAGAAGAAGATCCTTGACATT
TRCN0000060490   GTACTTTATGATAGGTGGGAT
TRCN0000060491   CCAGAAAGTTCACAACGGCAT
TRCN0000060492   GTCTAGAACGTCTGGAGATTT

BNIP1
TRCN0000033529   CCGGATCTGTAACCAAGAGAT
TRCN0000033530   CGAACGATCCTGGATGCAAAT
TRCN0000033531   CGTGATTGTTCAGGACCCTTA
TRCN0000033532   CTTCTCATCTTCCTTGCGCTA
TRCN0000033533   GCAATCGACAATCTAGAGAAA

BET1L
TRCN0000043218   TGGGTGGTCAAATCACCACTT
TRCN0000043219   GCCCTGGACATCGATAGGGAT
TRCN0000043220   CTGTGTGATCTTGCTGGGATT

TRCN0000043221   CGGGAGAACAAGCGAATGGCT
TRCN0000043222   CGGTACCTGGATGGCATGGTA

VTI1A
TRCN0000043358   GCTACAAACAAGAAATGGGAA
TRCN0000043359   GATACCAAATAGCAGTGGAAA
TRCN0000043360   GTGGAGAAACAGCTTGAAGAA
TRCN0000043361   CGAGCAAATTGGTCAGGAGAT
TRCN0000043362   GCAGAGATCACCAGCAAGATT

VTI1B
TRCN0000043338   GAAGAAATTGATCAGGGATTT
TRCN0000043339   CCTGGTTTACTACAAATTCTT
TRCN0000043340   GCAGGGCACTGAAAGCCTGAA
TRCN0000043341   GCTGCTGCTTTCCATTATCAT
TRCN0000043342   GAGACATGAAATATGGCATAT

c) SNARE-LIKE PROTEINS

SEC22L2 (SEC22A)
TRCN0000059543   CCAGTGTTATTTACTTGTCTA
TRCN0000059544   GCAGGAGTGCAGAAAGTATTT
TRCN0000059545   GCTGGCGGAATGTCAAATCTT
TRCN0000059546   CTGAATTTAATTCGAGGCTTT
TRCN0000059547   GCTTCTACTGATTATGAACAA

SEC22L3 (SEC22C)
TRCN0000059548   GCTCCTAATTTCCGAATGGAA
TRCN0000059549   CTATGTAAGTTCCTCTCAGAT
TRCN0000059550   GAAGGTTGTGACTTTAGTATA
TRCN0000059551   GTTCCTTTCGTAGCCTGCATT
TRCN0000059552   GAGTTCACCTTGCAGAACATT

B) OTHER MOLECULES

ANXA2
TRCN0000056143   CCTGCTTTCAACTGAATTGTT
TRCN0000056144   GCAGGAAATTAACAGAGTCTA
TRCN0000056145   CGGGATGCTTTGAACATTGAA
TRCN0000056146   CCAGAAAGTATTTGATAGGTA
TRCN0000056147   CTGTACTATTATATCCAGCAA

ARFGEF2
TRCN0000047313   GCTTCATTTCTATCATTCCAT

TRCN0000047314   CGCCAGTCTTTAAGCAGCATA
TRCN0000047315   CGACACCATTAAGACGCTTAT
TRCN0000047316   CCATTAAAGAAGCAGCGGAAA
TRCN0000047317   CCCTGAGCAATTTGAGGTCAT

SMPD3
TRCN0000048943   CCCAACAAGTGTAACGACGAT
TRCN0000048944   GTGGAGATTTCAACTTTGATA
TRCN0000048945   CCAAAGAATCGTCGGGTACAT
TRCN0000048946   CCCTTTGCGTTTCTCGGCTTT
TRCN0000048947   CGCATCGACTACATGCTGCAT

TSAP6 (STEAP3)
TRCN0000064643   CGGCAGAAACTTGTCTCATAA
TRCN0000064644   CGTCTGCTTCTATGCCTACAA
TRCN0000064645   CTTCAGCATCGTGAGTCCAAT
TRCN0000064646   GTCCATTGCAAACTCGCTCAA
TRCN0000064647   CAGCGATAGTAGCCTTGCCAA

CIT
TRCN0000006313   CCCTCCTATTAGAGTACGAAA
TRCN0000006314   GCCAATAAACTTGCAGCAAAT
TRCN0000006315   GCGTCCTCATACCAGGATAAA
TRCN0000006316   CGGAAGTATTCCGACACCATA
TRCN0000006317   GCTGATCTACTGAAGACAGAA

DGKA

TRCN0000006078   CCTCACAGTATTTATTATCCT
TRCN0000006079   CGGCCAGAAGACAAGTTAGAA
TRCN0000006080   GCTCTGGAAGTTCCAGTATAT
TRCN0000006081   GCTAAATATGTCCAAGGAGAT
TRCN0000006082   CCAAATCTATACCAAGCTCAA

PLD2
TRCN0000051148   CCAAGAAGAAATACCGTCATT
TRCN0000051149   GCCAAGTACAAGACTCCCATA
TRCN0000051150   CCGAAAGATATACCAGCGGAT
TRCN0000051151   CCTCTCTCACAACCAATTCTT
TRCN0000051152   CGATGAGATTGTGGACAGAAT

ATP6V0A1
TRCN0000038429   CCAGGATTGATGATCTCCAAA
TRCN0000038430   CCTGGAACTGACCGAATTAAA
TRCN0000038431   GCCGTCAGTATTTGAGGAGAA
TRCN0000038432   CCCTGAATATCTACTTTGGAT
TRCN0000038433   CCCAGAGTCTGGTTATTCAAT

ATP6V0A2

TRCN0000043493	CCCAGCATTCTGATTGAATTT
TRCN0000043494	CGTAAGTTCTTTCCAAAGAAA
TRCN0000043495	CCCAGACTAAATCAGTCACAA
TRCN0000043496	GCCACAAGGTTAAGAAGATAT
TRCN0000043497	CGGAGTGGCTACACACTTATA

ATP6V0A3 (TCIRG1)

TRCN0000038634	AGACGCTTTGTGGTTGATGTT
TRCN0000038635	CCGCAAGATCACGGACTGCTT
TRCN0000038636	CCTCGTGTTCCTAGTCATCTA
TRCN0000038637	CCACAAGATGAAGGCCGTGTA
TRCN0000038638	CAACTCCTTCAAGATGAAGAT

ATP6V0A4

TRCN0000038644	CCTGTGGATTTGATACGACTT
TRCN0000038645	CCCACATTTAACAGGACCAAT
TRCN0000038646	CCAAAGTTTCTTTGTGGTTAT
TRCN0000038647	GCCTGCATATATGACCGGAAA
TRCN0000038648	GCTCTGGACTATGGTGATGAA

C) RAB5 ISOFORMS

RAB5A

TRCN0000011215	GCAGCCTTCCTTTCCAAAGTT
TRCN0000007974	CCAGGAATCAGTGTTGTAGTA
TRCN0000007975	CTGGTCAAGAACGATACCATA

RAB5B

TRCN0000048229	GCCAGTCCTAGCATCGTTATT
TRCN0000048228	CCAAGCTGCAATCGTGGTTTA
TRCN0000048231	ACAGCTATGAACGTGAATGAT

RAB5C

TRCN0000072933	CCACCATGATTTCTCCATATA
TRCN0000072936	GAACGAAATCTTCATGGCAAT
TRCN0000072937	CATCACCAACACAGATACATT