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Roberta Palmulli

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*Laboratoire : Institut Curie - UMR144 - CNRS –  
Structure et Compartiments Membranaires*

# **ROLE OF CD63 IN THE BIOGENESIS AND THE FUNCTION OF EXOSOMES**

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Thèse de doctorat de Biologie Cellulaire

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## Abbreviations

<b>A<math>\beta</math></b>	Amyloid- $\beta$ peptide
<b>ACAT</b>	Acyl-CoA:cholesterol acyltransferase
<b>AD</b>	Alzheimer's disease
<b>ALIX</b>	ALG-2-interacting protein X
<b>ApoE</b>	Apolipoprotein E
<b>AP</b>	Adaptor protein
<b>APP</b>	Amyloid precursor protein
<b>ARF</b>	ADP-Ribosylation Factor
<b>ARMM</b>	Arrestin-domain-containing protein1 (ARRDC1) -mediated microvesicle
<b>Arp</b>	Actin-related protein
<b>BACE</b>	<i><math>\beta</math>-site APP cleaving enzyme</i>
<b>BLOC</b>	Biogenesis of lysosome-related organelles complex
<b>BMDC</b>	Bone marrow derived cell
<b>CAF</b>	Cancer associated fibroblast
<b>CCDC53</b>	Coiled coil domain containing protein 53
<b>CD9</b>	Cluster of differentiation 9
<b>CD63</b>	Cluster of differentiation 63
<b>CD81</b>	Cluster of differentiation 81
<b>CI-M6PR</b>	cation-independent mannose 6-phosphate receptor
<b>CLIC</b>	Clathrin- and dynamin-independent carrier
<b>CSC</b>	Cargo-selective complex
<b>DAG</b>	Diacylglycerol
<b>DCT</b>	Dopachrome tautomerase
<b>EBV</b>	Epstein-Barr virus
<b>ECM</b>	Extracellular matrix
<b>EE</b>	Early Endosome
<b>EEA1</b>	Early Endosome Antigen 1
<b>ELROs</b>	Endosome lysosome related organelles
<b>EMT</b>	Epithelial to mesenchymal transition
<b>ER</b>	Endoplasmic reticulum
<b>ERC</b>	Endosomal recycling compartment
<b>ERGIC</b>	ER-Golgi intermediate compartment
<b>ESCRT</b>	Endosomal sorting complex required for transport

<b>EV</b>	Extracellular vesicle
<b>FAM21</b>	Family with sequence similarity 21A
<b>GGAs</b>	Golgi-localized, $\gamma$ adaptin ear-containing ARF-binding proteins
<b>HDL</b>	high-density lipoprotein
<b>HMG-CoAR</b>	(3-hydroxy-3-methyl-glutaryl)-CoA reductase
<b>HPS</b>	Hermansky–Pudlak syndrome
<b>HRS</b>	Hepatocyte growth factor-regulated tyrosinase kinase substrate
<b>HSPG</b>	Heparan sulphate proteoglycans
<b>IDL</b>	Intermediate-density lipoprotein
<b>ILV</b>	Intraluminal vesicle
<b>JMY</b>	Junction mediating regulatory protein
<b>KLD</b>	Kringle-like domain
<b>LBPA</b>	Lyso-bisphosphatidic acid
<b>LDL</b>	Low-density lipoprotein
<b>LDLR</b>	Low-density lipoprotein receptor
<b>LE</b>	Late Endosome
<b>LMP1</b>	Latent membrane protein
<b>LROs</b>	Lysosome-Related Organelles
<b>LRP1</b>	Low-density lipoprotein receptor-related protein 1
<b>LXR</b>	Liver-X-receptor
<b>MARKS</b>	Myristoylated alanine-rich protein kinase C substrate
<b>MHC</b>	Major histocompatibility complex
<b>MVB</b>	Multivesicular Body
<b>MVE</b>	Multivesicular Endosome
<b>NPC</b>	Niemann-Pick type C
<b>NPF</b>	Nucleation promoting factor
<b>NTA</b>	Nanoparticle tracking analysis
<b>NTR</b>	N-terminal region
<b>ORP</b>	OSBP-related protein
<b>OSBP</b>	Oxysterol-binding protein

<b>PC</b>	Proprotein convertase
<b>PIKFyve</b>	Phosphoinositide Kinase, FYVE-Type Zinc Finger Containing
<b>PIP</b>	Phosphatidylinositol phosphate
<b>PKD</b>	Polycystic kidney disease domain
<b>PLD</b>	Phospholipase D
<b>PM</b>	Plasma membrane
<b>PS</b>	Phosphatidylserine
<b>PSEN</b>	Presenilin
<b>Rab</b>	Ras-associated binding protein
<b>RBP</b>	RNA-binding protein
<b>RE</b>	Recycling Endosome
<b>RPT</b>	Repeat domain
<b>SCAP</b>	SREBP-cleavage-activating protein
<b>SE</b>	Sorting Endosome
<b>SIMPLE</b>	Small integral membrane protein of the lysosome/late endosome
<b>SNX</b>	Sorting nexin
<b>SP</b>	Signal peptide
<b>SREBP</b>	sterol regulatory element binding protein
<b>START</b>	STAR (steroidogenic acute regulatory protein)-related lipid transfer
<b>STxB</b>	Shiga toxin B-subunit
<b>Syt VII</b>	Synaptotagmin VII
<b>TAM</b>	Tumor-associated macrophage
<b>TEC</b>	Tumor endothelial cells
<b>TEM</b>	Tetraspanin enriched microdomains
<b>TfR</b>	Transferrin receptor
<b>TGN</b>	Trans-Golgi Network
<b>Tsg101</b>	Tumor susceptibility gene 101
<b>TYRP</b>	Tyrosinase related protein
<b>VEGF</b>	Vascular endothelial growth factor
<b>VLDL</b>	Very low-density lipoprotein
<b>VLDLR</b>	Very low-density lipoprotein receptor
<b>Vps</b>	Vacuolar protein sorting
<b>WASH</b>	WASP and Scar homolog
<b>WAVE</b>	WASP and verprolin homolog

<b>WASP/N-WASP</b>	(Neuronal)-Wiskott-Aldrich Syndrome protein
<b>WCA</b>	WH2-connector-acid
<b>WHAMM</b>	WASP homolog associated with actin, membranes and microtubules
<b>WPBs</b>	Weibel-Palade bodies

## **Abstract**

Exosomes are 30 to 200 nm vesicles released by all cell types that contain specific sets of proteins, RNAs and lipids. The secretion and intercellular exchange of exosomes and their contents are involved in most physiological and pathological processes, such as the progression of metastatic cancers including melanoma.

The functional capacities of exosomes are directly related to their composition and to the molecular machineries that regulate their biogenesis. The aim of my thesis project was to study one of the biogenesis pathways of exosomes and to establish a functional relation between composition and function of exosomes, especially during the development of cutaneous melanoma.

Exosomes are generated as intraluminal vesicles (ILVs) in the lumen of multivesicular endosomes (MVEs). In these compartments, membrane proteins and lipids are either excluded from MVEs by external budding or are sequestered on ILVs by internal budding. Among the different sorting mechanisms that regulate the biogenesis of ILV I was interested in the one involving the tetraspanin CD63. Tetraspanins are proteins with four transmembrane regions that regulate the trafficking, cleavage and signaling of partner proteins by incorporating them into microdomains enriched in certain lipids such as cholesterol. CD63 is the only tetraspanin mainly located on ILVs of MVEs and is widely used as a marker for exosomes. Nevertheless, its role in the biogenesis of exosomes is still not well understood and has been the main focus of my research.

I was able to show that in the HeLa cell line, CD63 regulates the sorting of cholesterol on ILVs and exosomes. In the absence of CD63, cholesterol is excluded from MVEs by an actin-dependent outward budding mechanism to be addressed to other cellular compartments. In the pigmented melanoma cell line MNT-1, I confirmed this mechanism and showed its physiological relevance in the sorting of endogenous apolipoprotein E (ApoE) to ILVs, a necessary process in the early stages of pigmentation.

Interestingly, the expression of ApoE and CD63 is associated with an inhibition of the metastatic capacities of melanomas. In parallel, I studied the relevance of this sorting process on the pro-metastatic properties of melanoma exosomes by focusing on their potential interaction with the extracellular matrix. My work shows that melanoma cells secrete subpopulations of exosomes with distinct composition. ApoE enrichment of one of these subpopulations is directly correlated with their inability to physically interact with collagen. Finally, we tested the hypothesis that the exosome-collagen interaction could influence the migration capacity of melanoma cells.

Overall, my data establish CD63 as a regulator of the balance between import and export processes in MVEs that contribute to endosomal homeostasis. In particular, my research shows that CD63 determines the composition of exosomes by regulating cholesterol (and ApoE) sorting in ILVs and exosomes and links this sorting mechanism to the ability of exosomes to interact with the extracellular environment.

## Résumé

Les exosomes sont des vésicules de 30 à 200 nm libérées par tous les types cellulaires qui contiennent des sets spécifiques de protéines, d'ARN et de lipides. La sécrétion et l'échange intercellulaire des exosomes et de leurs contenus sont impliqués dans la plupart des processus tant physiologiques que pathologiques comme la progression de cancers métastatiques tels que le mélanome.

Les capacités fonctionnelles des exosomes sont directement liées à leur composition et aux machineries moléculaires qui régulent leur biogenèse. Le but de mon projet de thèse a été d'étudier une des voies de biogenèse des exosomes et d'établir une relation fonctionnelle entre composition et fonction des exosomes en particulier lors du développement du mélanome cutané.

Les exosomes sont générées sous forme de vésicules intraluminales (ILV) dans la lumière des endosomes multivésiculaires (MVE). Dans ces compartiments, les protéines et lipides membranaires sont soit exclus du MVE par bourgeonnement externe soit séquestrés sur les ILV par bourgeonnement interne. Parmi les différents mécanismes de tri qui régulent la biogenèse de l'ILV je me suis intéressée à celui impliquant la tétraspanine CD63. Les tétraspanines forment une famille de protéines à quatre domaines transmembranaires qui régulent le trafic, le clivage et la signalisation de protéines partenaires en les incorporant dans des microdomaines enrichis en certains lipides comme le cholestérol. CD63 est la seule tétraspanine principalement localisée sur les ILVs des MVEs et elle est largement utilisée comme marqueur d'exosomes. Néanmoins, son rôle dans la biogenèse des exosomes n'est toujours pas bien compris et a été l'objet principal de mes recherches.

J'ai pu montrer que dans la lignée cellulaire HeLa, CD63 régule le tri du cholestérol sur les ILVs et les exosomes. En l'absence de CD63, le cholestérol est exclu des MVEs par un mécanisme actine-dépendant de bourgeonnement externe pour être adressé à d'autres compartiments cellulaires. Dans la lignée cellulaire de mélanome pigmentée MNT-1, j'ai confirmé ce mécanisme et montré sa relevance physiologique dans le tri de l'apolipoprotéine E endogène (ApoE) aux ILVs, un processus nécessaire aux premières étapes de la pigmentation.

De façon intéressante, l'expression d'ApoE et de CD63 est associée à une inhibition des capacités métastatiques des mélanomes. En parallèle, j'ai étudié la relevance de ce processus de tri sur les propriétés pro-métastatiques des exosomes de mélanomes en me focalisant sur leur potentielle interaction avec la matrice extracellulaire. Mes travaux montrent que les cellules de mélanome sécrètent des sous-populations d'exosomes à la composition distincte. L'enrichissement d'une de ces sous-populations en ApoE est directement corrélé à leur incapacité à interagir physiquement avec le collagène. Enfin, nous avons testé l'hypothèse selon laquelle l'interaction exosomes-collagène pouvait influencer les capacités migratoires des cellules de mélanome.

Globalement, mes données établissent CD63 comme régulateur de la balance entre les processus d'import et d'export dans les MVEs qui contribuent à l'homéostasie endosomale. En particulier, mes recherches montrent que CD63 module la composition des exosomes en

régulant le tri du cholestérol (et de l'ApoE) dans les ILV et les exosomes et lient ce mécanisme de tri à la capacité des exosomes à interagir avec l'environnement extracellulaire.

# **INTRODUCTION**

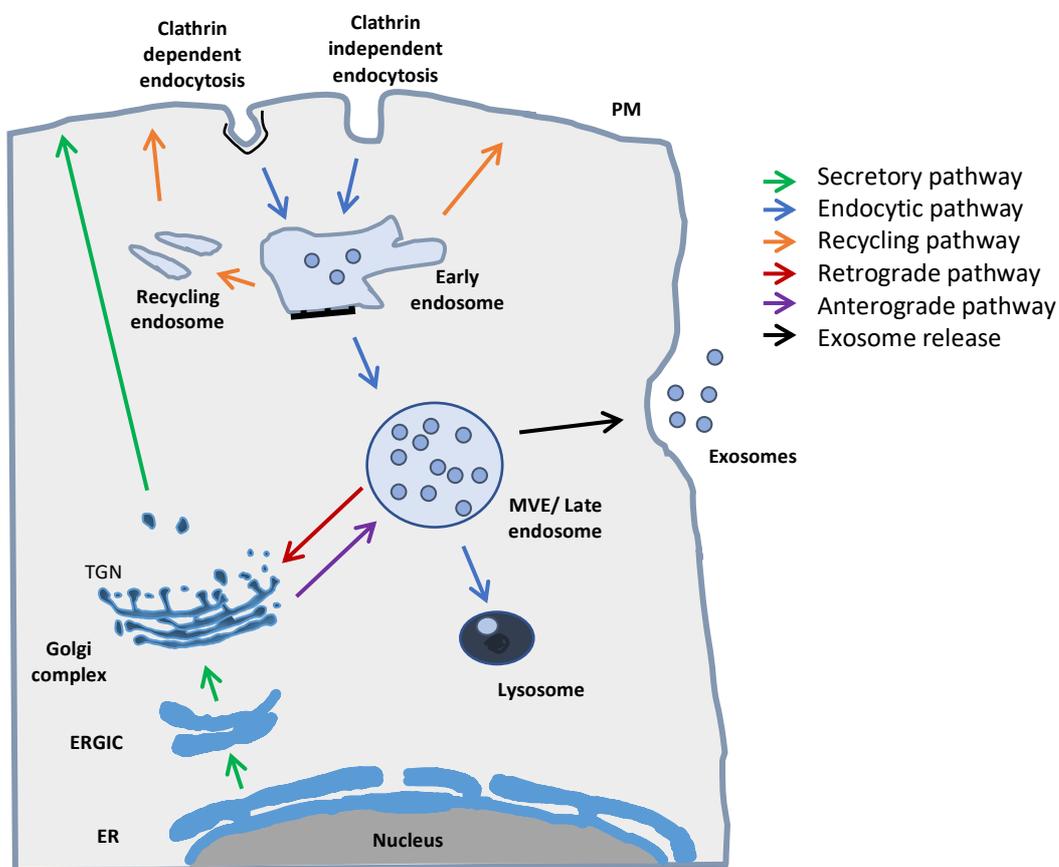


# INTRODUCTION

## 1. Intracellular trafficking and endosomal dynamics

### 1.1. Overview of intracellular trafficking pathways

Eukaryotic cells contain membrane-bound organelles that communicate with each other to exchange proteins and lipids and to secrete them in the extracellular environment. Two main intracellular trafficking pathways can be identified: the secretory pathway, for the transport of cargoes from the cell to the extracellular environment, and the endocytic pathway for the import of cargoes from the extracellular environment (**Figure 1**).



**Figure 1.** Overview of the main intracellular trafficking pathways.

Cargo proteins synthesized in ribosomes are first transported to the endoplasmic reticulum (ER) for their correct folding, and then to the Golgi apparatus through the ER-Golgi intermediate compartment (ERGIC). Inside the Golgi apparatus proteins are transported from the cis-Golgi cisterna through the medial -Golgi and the trans-Golgi cisterna until they reach the Trans-Golgi Network (TGN) [1]. In these compartments, proteins can undergo

further modifications, such as glycosylation. In the TGN, cargo proteins are segregated and incorporated in vesicles that will eventually reach their final destination [2], which can be the extracellular space, the plasma membrane (PM) or the endosomal system (anterograde pathway) (Figure 1).

The endocytic pathway plays an important role in regulating the fate of membrane and extracellular molecules [3]. From a classical point of view, the endocytic pathway delivers cargoes to lysosomes for their final degradation (degradative pathway), but cargoes reaching the endosomal system after endocytosis can also escape lysosomal degradation through recycling to the plasma membrane (recycling pathway), by being delivered to other cellular compartments such as Golgi apparatus (retrograde pathway), or by being secreted through exosomes (Figure 1).

Therefore, the secretory pathway and the endocytic pathway are interconnected and tightly regulate the fate of a given cargo. Despite the diversity of these pathways, transport mechanisms employ similar processes including the formation of vesicular or tubular carriers that will bud from the membrane of origin, be transported and fuse with the membrane of the acceptor compartment [4]. Additionally, membranes from two different organelles can establish non fusogenic contacts, called membrane contact sites (MCS), that contributes to cargo exchange in between two organelles. This pathway is particularly important for the exchange of lipids such as cholesterol and phosphoinositides (PIPs).

## **1.2. The endosomal system**

The endosomal system is composed by a continuum of compartments that acquire different properties and morphology during their maturation. The identity of these different stages is maintained by their content and by the specific composition of their membranes in terms of lipids, such as phosphoinositides, and proteins, such as the Rab GTPases.

In a simplified view, the endosomal system is composed by early endosomes (EE), multivesicular endosomes (MVE) and late endosomes (LE), and lysosomes [5].

### **1.2.1. Early Endosomes**

Endocytosis is the main pathway by which cells can control the composition and organization of their plasma membrane and internalize molecules from the extracellular environment [3]. Among the many endocytosis pathways that have been described, the best characterized is clathrin-dependent endocytosis, which requires the formation of a clathrin-coated pit, followed by the scission of these vesicles, in a process mediated by the GTPase dynamin [6]. The so-called “clathrin independent endocytosis pathways” include caveolae-mediated endocytosis (that also requires dynamin) and the clathrin- and dynamin-independent carrier (CLIC) tubular intermediates [7], which formation involves small GTPases such as the ADP-ribosylation factor 6 (ARF6) and Rho subfamily members RhoA and Cdc42. All types of endocytic vesicles will fuse with early endosomes and deliver their content. Early endosomes appear as vacuolar compartments (sorting endosomes, SE) from which thin tubular structures emanates to form the so-called recycling endosomes (RE) [8].

EE are characterized by a Ph of 6.5 and by the presence of the GTPase Rab5 and its effector early endosome antigen 1 (EEA1) and of the phosphoinositide PI3P [3]. Rab5 localizes in nano-domains of the endosomal membrane [9] and is a key regulator of early endosome formation, fusion, maturation and dynamics [10]. In addition, the GTPase Rab4 is also localized at EE [11] where it contributes to the fast recycling of cargoes (e.g. transferrin receptor, TfR) to the plasma membrane [12]. RE tubules can also detach from EE to form the endosomal recycling compartment (ERC), which appears as a collection of recycling endosomal tubules with a perinuclear localization [8] or a more peripheral localization in some cell types (e.g. melanocytes) [13]. ERC is characterized by the presence of the GTPase Rab11 which is involved in the slow recycling of cargoes (e.g. TfR) to the plasma membrane [14]. In addition, recycling tubules emanating from EE also mediates the transport of cargoes to the Trans-Golgi Network (TGN), lysosomes or lysosome-related organelles (LROs) (see below).

SE is characterized by the presence of a flat bilayered clathrin coat [15]. The coat contains the ESCRT-0 proteins HRS and STAM and therefore it is involved in the sorting of cargoes to ILVs for lysosomal degradation [15], [16]. In addition, clathrin has been involved in the retrograde transport of Shiga toxin B-subunit (STxB), acting upstream of the retromer complex [17], [18] (and further discussed below).

Loss of Rab5 and acquisition of Rab7 together with PIP conversion and ILV formation will induce the maturation of EEs into LEs [5]. Interfering with EE maturation results in an enlargement of EEs. This phenotype has been observed in pathologies such as Alzheimer's disease and Down's syndrome, despite the molecular mechanisms involved need to be further investigated [19].

### **1.2.2. Multivesicular Endosomes and Late Endosomes**

LEs derive from the SE [20], they have a luminal pH of ~5.5 and appear as globular vacuoles containing ILVs, with the tubular extension of EEs being lost. For this reason, they are also called multivesicular endosomes (MVEs) or multivesicular bodies (MVBs). Rab5-Rab7 switch represent a fundamental step for LE biogenesis. While Rab7 recruitment is initiated by Rab5 GTP, the loss of Rab5 is not fully understood. The recruitment of Rab7 can initiate a cascade that induces the conversion of Rab5 to its GDP-bound form and the detachment of Rab5 from the endosomal membrane [21]. Alternatively, the Rab7 containing domain can undergo fission from EE originating endosomal carrier vesicles, which will eventually fuse with pre-existing late endosomes [22]. In addition, Rab GTPases associated with EE and recycling of cargoes to the plasma membrane (e.g. Rab4, Rab22) are lost while Rab9, that is involved in transport pathways between TGN and late endosomes, is acquired. Interestingly, recruitment of Rab9 seem to precede the recruitment of Rab7 [23]. The formation of F-actin patches on early endosome membranes, mediated by AnnexinA2, the Arp2/3 complex [24], the ERM protein moesin and the actin-binding protein cortactin [25] is also required for the maturation of MVEs. Other processes involved in endosome maturation are the conversion of PI3P into PI(3,5)P<sub>2</sub> mediated by the kinase PIKfyve, the acquisition of lysosomal proteins

such as acid hydrolases, mostly derived from the TGN, and the acquisition of SNARE proteins that will mediate the fusion of LE with each other or with lysosomes [5]. Finally, ILV formation, that was already started in EEs, continues in MVEs and depends on several mechanisms. The first described and most characterized mechanism of ILV biogenesis depends on the endosomal sorting complex required for transport (ESCRT). The ESCRT complex is composed of four different subcomplexes – ESCRT-0, -I, -II and -III – and associated proteins such as ALIX, VTA1 and ATPase VPS4 [26] that act together in a sequential manner. The first step consists in the recruitment of ESCRT-0 (in particular HRS) at the endosomal limiting membrane. This process is mediated by the ubiquitin moieties attached to cysteine residues present in the cytoplasmic domain of transmembrane cargo proteins that have to be sorted [16] and by PI3P [27]. ESCRT-0 and ESCRT-I then cluster the cargo proteins under the flat clathrin coat, recruited by ESCRT-0 [28], creating a subdomain of the endosomal membrane that buds into an ILV. The flat clathrin coat is thought to avoid cargo protein diffusion and their targeting to alternative pathways such as recycling or retrograde transport [29]. The clathrin coat seem also to regulate ESCRT-0 dissociation and to induce membrane deformation [28]. The clathrin coat is then removed, potentially by the V-ATPase HSC70 [30], [31], in concomitance with the recruitment of ESCRT-II and -III that will mediate membrane scission and ILV formation together with VPS4 [32]. At the same time, deubiquitinating enzymes that remove the ubiquitin tag from cargo proteins [33], [34] are recruited, before the release of the newly forming ILVs into the lumen of MVEs. Alternatively, ESCRT-independent pathways have been described and they involve proteins such as syntenin or tetraspanins and lipids such as ceramide (see below). Finally, the fate of an MVE is to fuse with lysosomes and degrade their content, alternatively MVEs can fuse with the plasma membrane and release their ILVs as exosomes in the extracellular space [35] (see below).

### **1.2.3. Lysosomes**

Lysosomes usually appear as globular organelles with an electron dense lumen and irregular membrane sheets. They contain acid hydrolases that are activated at acidic Ph (~4.5) and contribute to the degradation of the endosomal content to generate molecules that can be used as nutrients by the cell [8]. Lysosomes can deliver their content to LE by kiss and run events before full fusion with LE to form an endolysosome. Endolysosomes are now considered as the site where hydrolases are activated and where degradation occurs. On the contrary, terminal storage lysosomes are non-hydrolytic stores of acid hydrolases [36]. In addition, lysosomes can fuse with autophagosomes to form an autolysosome and degrade their content. Beside degradation, lysosome play additional functions such as sensing of nutrient availability, and they can undergo fusion with the plasma membrane and contribute to plasma membrane repair [37]. Reformation of terminal storage lysosomes from endolysosomes is mediated by membrane tubulation events that require the kinase PIKfyve among other mechanisms [38][39].

Lysosomal activity is impaired in lysosomal storage diseases, which are mostly caused by enzyme deficiencies within the lysosome resulting in the accumulation of undegraded substrates, and in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [40].

#### **1.2.4. Lysosome related organelles**

The endocytic pathway described above is present in all cells, however some cell types have adapted and developed their endosomal system in order to fulfill specific functions. In particular, some cell types contain specialized lysosomes called lysosome-related organelles (LROs) that share features with lysosomes, such as their low pH or the presence of LE/lysosomal proteins, as exemplified by the tetraspanin CD63. LROs include melanosomes in pigment cells, dense and alpha granules in platelets, Weibel-Palade bodies (WPBs) in endothelial cells and lytic granules in cytotoxic T cells among others. While in some cell type LROs can be considered as specialized lysosomes, in other cell types, such as melanocytes, they coexist with conventional lysosomes [41]. LROs derive directly from the endocytic pathway, as in the case of melanosomes, or from the TGN secretory pathway and acquire some content from the endolysosomal system, as in the case of WPBs, and they undergo secretion in most of the cases. Taking into account that LROs share features with early/late endosomes, more than with lysosomes in some cases, and that they coexist with conventional lysosomes, Delevoe et al. recently proposed a new definition of LROs as endosome lysosome-related organelles (ELROs) [42]. Finally, ELROs are all affected in genetic disorders such as the Hermansky-Pudlak (HPS), Chediak-Higashi, Griscelli syndromes and familial haemophagocytic lymphohistiocytosis, which result from the loss of function of endo/lysosomal proteins involved in ELRO biogenesis and/or secretion.

Among all ELROs, one of the most studied and characterized is the melanosome in pigmented cells. In skin melanocytes, melanosomes are the site where melanin pigment is synthesized and stored before being transferred to the neighbor keratinocytes where melanin determines the skin color and contribute to the protection from UV radiation.

In this work, melanocytes were used as model to investigate intracellular trafficking pathways that contribute to exosome secretion and melanosome biogenesis; therefore, a more detailed description of these pathways will be found later in this manuscript.

#### **1.3. Intracellular trafficking pathways involving the endosomal system**

As introduced before, newly synthesized cargoes can be transported directly from the secretory pathway (ER-Golgi-TGN) to the endosomal system, following the so-called anterograde transport. Alternatively, transmembrane proteins can be first transported to the PM and then reinternalized by endocytosis. On the contrary, cargoes, independently of their origin, can exit the endosomal system in order to escape degradation. These retrieving pathways are referred as recycling pathways for endosome-PM transport or retrograde transport in the case of endosome-TGN transport.

### **1.3.1. Anterograde transport**

The direct transport of protein from the Golgi apparatus to the endosomal system has been mostly studied for the transport of endo/lysosomal resident proteins (e.g. endo/lysosomal transmembrane proteins, acid hydrolases). Transmembrane proteins present a small amino acid motif, tyrosine or dileucine-based motif, in their cytosolic region that is recognized by clathrin adaptor proteins, allowing the recruitment of clathrin and the formation of a clathrin-coated vesicle that will eventually fuse with the endosome [43]. Recruitment of these adaptors is not only mediated by the cargo recognition but also by the phospholipidic composition of the Golgi membranes in particular PI4P [44], [45]. Adaptor proteins include adaptor protein family complexes (AP), ADP ribosylation factor (ARF) and Golgi-localized,  $\gamma$  adaptin ear-containing ARF-binding proteins (GGAs). AP family includes AP-1, AP-2, AP-3, AP-4 and AP-5. While AP-2 mediates clathrin dependent endocytosis at the PM [6], AP-1, AP-3 and AP-4 localize at the Golgi. AP-1, AP-3, AP-4 [46] and AP-5 [47] are also located in endosomes. AP-1 and GGAs function together to regulate the anterograde transport of the cation-independent mannose 6-phosphate receptor (CI-M6PR) [48] and sortilin [49], two transmembrane receptors for soluble lysosomal acid hydrolases. They exit the TGN in the same clathrin coated vesicles [50] to be transported to early endosome and, after the release of their cargoes, they are recycled back to the TGN following the retrograde pathway. Alternatively, the low-density lipoprotein receptor-related protein 1 (LRP1) has been implicated in the transport of acid hydrolases in absence of M6PR or sortilin [39].

Lysosomal membrane proteins such as LAMP-1, LAMP-2, CD63 (also called LAMP-3) and LIMP-2 are also transported from the TGN directly to endosomes mostly through AP-3 vesicles [52], but they can also transiently arrive to the PM and be reinternalized by AP-2 [48]. Finally, clathrin and AP-1 independent carriers, positive for the SNARE protein VAMP7 and hVps41, are involved in the direct transport of LAMP-1 and LAMP-2 from the TGN to late endosomes [53].

### **1.3.2. Retrograde transport and recycling transport**

As already mentioned, receptors such as M6PR and sortilin exit the endosomal system to be transported back to the TGN. Several molecular pathways have been involved in this retrograde transport. Among them, the best characterized pathway is regulated by the retromer complex [54]. The retromer complex was first described in yeasts where it mediates the retrieval of the vacuolar protein Vps10p [55]. In mammals the complex consists in two subcomplexes: the trimer Vps26/Vps29/Vps35 and a membrane-bound dimer of sorting nexins (SNXs), SNX1/2 and SNX 5/6, also called SNX-BAR proteins. While the trimer is involved in cargo recognition (cargo-selective complex, CSC), the SNX dimer induces membrane curvature through their BAR domain, to allow the formation of transport tubular carriers. Nevertheless, SNX-BAR proteins can function independently of the CSC [56], [57]. In the context of retrograde transport, SNX1 has been shown to mediate the retrograde transport of sortilin through short, non-branched vesicles and tubules, emanating from early endosomes, that may not necessary require the presence of the CSC [50]. The recruitment of

the retromer complex on endosomal membranes is mediated by several factors, including the affinity of the Phox homology (PX) domain of SNX1 for PI(3)P [58], the small GTPase Rab7a, [59], [60] and SNX3 [61], another sorting nexin involved in the endosome-TGN transport of Wntless [62] independently of the SNX-BAR dimer. Despite retromer has been observed on early endosomes, it is likely that active retrograde transport takes place in maturing endosomal compartments [48].

In addition to the retromer complex, the small GTPase Rab9 and its effectors TIP47 and p40 have been involved in retrograde transport of M6PR from late endosomes [63], [64], [65, p. 9]. While the retromer complex is associated with tubular structures emanating from the SE, Rab9 is associated with vesicles budding from late endosomes [66]. The newly identified Rab GTPase Rab7b has also been involved in endosome to Golgi transport, probably in concert with Rab9, despite the respective role of Rab7b and Rab9 is not clear and need to be further investigated [67]. Other pathways mediating retrograde transport endosomes-TGN involve the adaptor proteins AP1 [68] and AP-5 [69], the phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) [70]. The kinase PIKfyve is part of the “PIKfyve complex” together with the phosphatase FIG4 and the scaffold protein Vac14. Interestingly, PIKfyve has been shown to interact with the Rab9 effector p40 [71] while Vac14 can interact with Rab9 and the Rab7 GAP TBC1D15 [72], that also negatively regulates the recruitment of the retromer complex [60]. Overall, these observations suggest that the different pathways can be interconnected. Apart from its role in endosome-TGN retrograde transport, the retromer complex has been implicated in the recycling of cargoes to the plasma membrane. In this case the trimer Vps26/Vps29/Vps35 interact with the SNX-FERM protein SNX27. In particular, the interaction is mediated by the PDZ domain of SNX27 with Vps26 [73]. This complex participates to the recycling of the glucose transporter GLUT1 and the  $\beta$ 2-adrenergic receptor  $\beta$ 2AR [73], [74, p. 27]. Another SNX-FERM member, SNX17 is involved in the recycling to the plasma membrane of cargoes such as LRP1 and the integrin  $\alpha$ 5 $\beta$ 1 together with the retriever complex. This complex is composed by the proteins Vps29, C16orf62, a distant homolog of Vps35, and DSCR3, that has similarities with Vps26 [75], [76]. Finally, another complex called CCC and composed by the trimer COMMD, CCDC22 and CCDC93, cooperate with retromer/SNX27 retriever/SNX17 for the recycling of specific cargoes [75].

#### **1.4. Formation of transport carriers**

A common feature of all intracellular trafficking pathway is the formation of transport carrier.

The formation of vesicular carriers comprises three main steps: the recognition of the cargoes that have to be retrieved, the formation and elongation of a tubule and the scission of the tubule. Once formed, these tubular/vesicular carriers are transported toward the acceptor compartment with which they will fuse to release their content. These steps are briefly described below. In addition, the role of the actin cytoskeleton in some of these steps is described.

#### 1.4.1. Cargo recognition

Cargo recognition is mediated by the presence of specific sorting motifs, such as the tyrosine-based or dileucine-based motifs recognized by APs and GGAs.

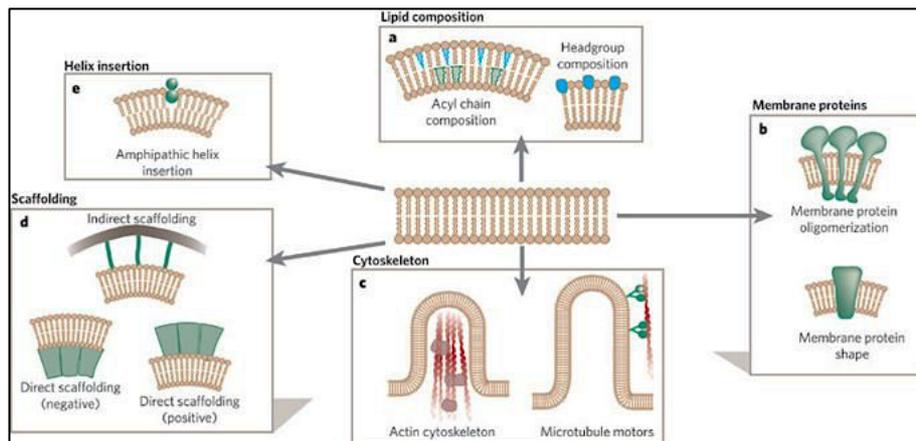
The retromer complex binds a variety of cargoes and several recognition motifs have been described [54]. Vps35 can be considered as the main cargo-binding subunit, as it recognizes binding motifs in receptors such as M6PR and sortilin, but cargo binding with Vps26 has also been reported [77]. Recently SNX-BAR proteins have been involved in cargo recognition of CI-M6PR and in the regulation of its sorting independently of the retromer trimer [78], [79]. The recently determined structure of the membrane-assembled retromer coat by cryo-electron tomography illustrate how Vps5 (yeast analogue of SNX-BARs) is directly associated to tubular membranes from which arches of the trimer Vps26/Vps29/Vps35 extend, away from the membrane surface [80]. This observation suggests that, in addition to their role in membrane curvature sensing, SNX-BAR proteins would contribute to cargo recognition together with the CSC, and that the CSC would also act as a scaffold complex for the maintenance and elongation of the tubule.

In the context of endosome-plasma membrane transport, the PDZ domain of SNX27 allows the binding of PDZ-binding domain containing cargoes (e.g. GLUT1 and  $\beta$ 2AR) independently of the retromer complex, although the interaction with Vps26 enhances the affinity of SNX27 with the cargoes [73], [75]. Similarly, the FERM domain of SNX17 recognize the NPxY/NxxY motif in cargoes such as the integrin  $\alpha$ 5 $\beta$ 1 [76].

After recognition, the cargoes are clustered in subdomains that will bud in order to form a vesicle or a tubule.

#### 1.4.2. Membrane deformation, elongation and fission

The formation of a bud is initiated by the induction of a positive membrane curvature through several mechanisms [81] (**Figure 2**). First of all, the lipidic composition of the membrane can induce curvature by itself, as exemplified by lysophosphatidic acid and phosphatidic acid [82]. In addition, lipids such as phosphoinositides contribute to the recruitment of proteins that sense and induce membrane curvature (e.g. BAR domain containing proteins). Indeed, several families of proteins can deform a membrane by acting as a scaffold around it. Among them we can distinguish coat proteins, such as clathrin and COPI or COPII (mediators of vesicular trafficking at the ER-Golgi interface), that polymerize creating a curved structure around the membrane, or the BAR domain containing proteins (e.g. SNX1/2 and SNX 5/6) that preferentially bind to curved membranes and sense membrane curvature. BAR domains are often found in proximity of N-terminal amphipathic helices (called N-BAR domains) that also contribute to membrane curvature by inserting their amphipathic helices in the membrane leaflet [81], [83]. In addition, the clustering of transmembrane proteins with conical shape can induce membrane curvature, as shown for caveolin [84] or flotillin [85]. Finally, the polymerization of the actin cytoskeleton together with the pulling forces exerted by molecular motors on microtubules also contributes to membrane bending and tubule elongation.



**Figure 2. Mechanisms of membrane deformation.**

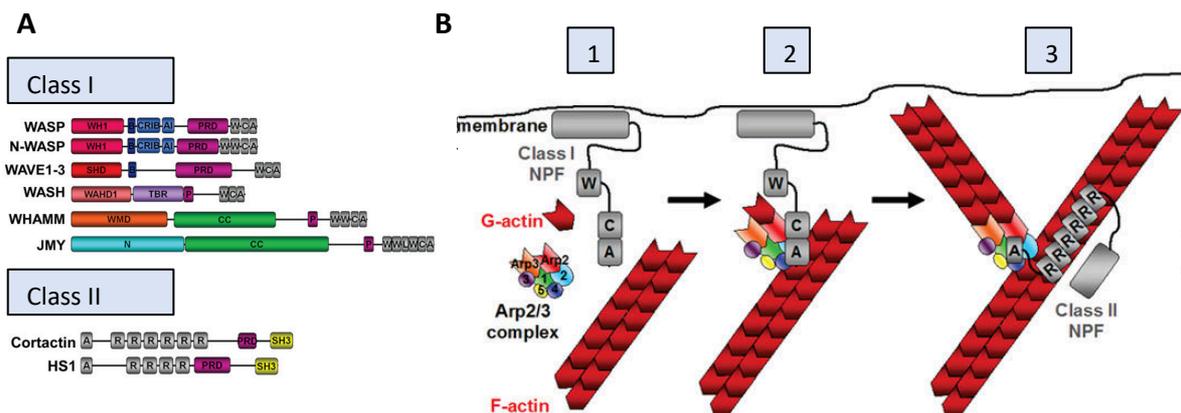
Different mechanisms can promote membrane curvature: (a) change in lipid composition of the membrane, (b) clustering of transmembrane proteins, (c) forces exerted by the cytoskeleton, (d) scaffolding by proteins such as coat or BAR domain proteins, (e) insertion of amphipathic helix into the membrane leaflet. From (McMahon & Gallop, 2005).

After the formation of the initial bud, the membranes undergo elongation and change their shape in order to create a spherical carrier (vesicle), as in the case of endocytosis, or a cylindrical carrier (tubule), as in the case of endosomal recycling [86]. Elongation of the vesicle/tubule is driven along microtubules by molecular motors such as kinesins and dyneins [86] and is regulated by proteins such as the BAR domain containing proteins that can act as scaffold proteins and stabilize the tubules.

Finally, membrane fission is required to generate the transport carrier. This process consists in the constriction of the neck up to the hemifusion of the membranes that will generate the final carrier. Membrane fission is not only regulated by the forces exerted by the cytoskeleton but also by protein machineries [87]. Among them we can identify dynamin, a large polymeric protein that assemble around the neck of endocytic vesicles, small GTPase (e.g. Arf1) as in the case of COPI vesicles, and ESCRT-III, that assemble in a spiral-like structure to promote ILV budding. A recent study *in vitro* shows that nonspecific protein crowding can also promote membrane fission [88]. In addition, the lipid composition of the membrane may also contribute to membrane fission. Finally, recent studies illustrate the role of the ER in the fission of endosomal tubules. ER forms contact sites with endosomes at constricted sorting domains. These contacts appear to limit free diffusion of cargoes in the membrane and to spatially and temporally regulate the fission of endosomal tubules [89]. The formation of MCS at the site of tubule fission is mediated by the interaction of endosomal SNX2 with the ER protein VAP-A, that in turn regulates PI4P levels on endosomes, and WASH-mediated actin polymerization to allow tubule fission and correct cargo recycling [90]. One last study shows that endosomal fission is driven by the interaction of the microtubule-severing enzyme spastin and the ESCRT protein IST1 at ER–endosome contacts and that this process not only controls M6PR trafficking but also contributes to the maintenance of lysosome morphology [91].

### 1.4.3. Role of the actin cytoskeleton

Actin cytoskeleton originates from the polymerization of an ATP-binding protein, the globular actin, that undergo self-assembly to create filamentous actin (F-actin). Because self-assembly is a slow and kinetically unfavorable process, actin polymerization is promoted and regulated by several factors. Among them we can distinguish the Arp 2/3 complex, which induce the formation of branched actin, and the formins, which generate linear unbranched F-actin filaments [92]. Here I will focus mostly on the role of the Arp 2/3 complex, which is particularly important in the formation of transport carriers and in the regulation of many endosomal processes. The Arp 2/3 complex is composed by seven subunits: ARP2 and ARP3 plus five additional subunits, ARPC1–ARPC5. The complex binds to the side of an existing actin filament and initiates the assembly of a new filament, which will have an  $\sim 70^\circ$  Y-branch inclination compared to the main filament. The activity of the Arp 2/3 complex is regulated by the so-called nucleation-promoting factors (NPFs). Class I NPFs use a carboxy-terminal WCA domain to promote actin nucleation and comprise several proteins such as WASP, N-WASP, WAVE, WASH, WHAMM and JMY [93]. Class II NPF include cortactin and its homolog in hematopoietic cells HS1 (Figure 3).



**Figure 3. Model for Arp2/3 mediated actin nucleation and branching.**

**A.** Structural domains of Class I and Class II NPFs. **B.** (1) The Arp2/3 complex is recruited to cellular membranes by Class I NPFs. (2) The WCA domain interacts with Arp2/3 and brings G-actin monomer to allow the nucleation of a new filament with a  $70^\circ$  Y-branch inclination. (3) Class II NPFs, such as cortactin stabilize branchpoints. (Adapted from Campellone & Welch, 2010).

While WASP is mostly expressed in the hematopoietic lineage, N-WASP is ubiquitously expressed. N-WASP is stabilized by WIP (WASP-interacting protein) [94] while it is activated by interaction with the small GTPase Cdc42 and with PI(4,5)P<sub>2</sub> [95]. WASP mostly localizes at the PM where it is involved in filopodia/invadopodia formation and in endocytosis. WAVE is also involved in processes at the PM such as lamellipodia formation and endocytosis [92]. WHAMM is mostly localized at the ER, ERGIC and Golgi where it is involved in membrane tubulation and transport of vesicles between the ER and the Golgi [96]. JMY promotes the

formation of branched F-actin by activation of Arp 2/3 or the formation of unbranched F-actin via its WH2 domains. In response to DNA damage JMY can translocate in the nucleus where it activates p53-dependent transcription [97], [98].

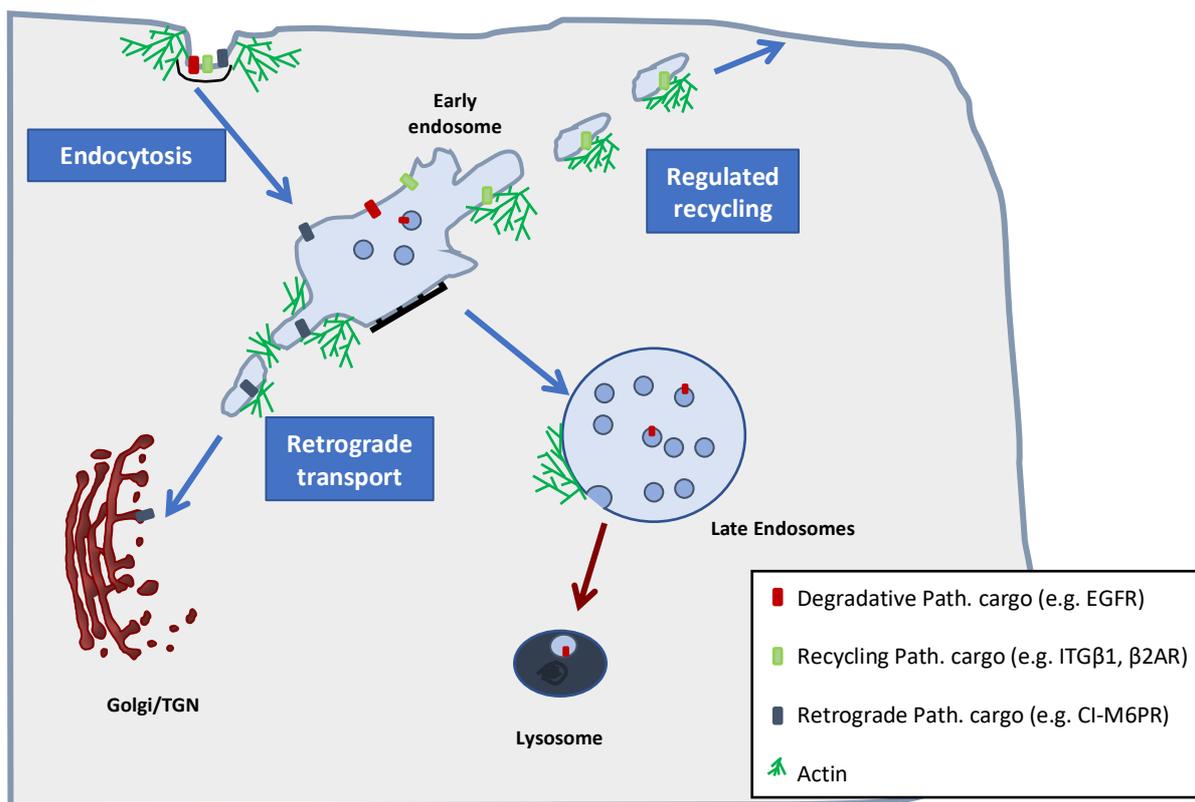
WASH is a multimeric assembly of five proteins: WASH1 (WASHC1), Strumpellin (WASHC5), family with sequence similarity 21A and C (FAM21A/C or WASHC2A/C), coiled coil domain containing protein 53 (CCDC53 or WASHC3), and the Strumpellin and WASH interacting protein (SWIP or WASHC4). WASH complex localizes on sorting and recycling endosomes where it contributes to endosomal fission [99]. Endosomal WASH participates in the recycling of cargoes to the TGN, as for M6PR [100] or to the PM, as in the case of Tfr, LDLR,  $\alpha 5\beta 1$  integrin or  $\beta 2AR$  [74], [99], [101], [102]. The activity of WASH is regulated by its ubiquitination and de-ubiquitination mediated respectively by MAGE-L2-TRIM27 [103] (also recruited by the retromer) and USP7 [104], that in turn finely tunes F-actin levels. The endosomal recruitment of the WASH complex is mediated by several factors including the retromer subunit Vps35 that interact with FAM21 tail [105]. A recent study shows that the recruitment of WASH can be also mediated by the ESCRT-0 protein HRS [106], despite the precise mechanism is not yet been defined. In addition to Vps35, WASH can be recruited to tubular domains involved in cargo recycling by SNX27 [73], SNX1 indirectly via RME-8 [107], or by the retriever complex indirectly via the CCC complex [108], [76]. It is likely that the recruitment and maintenance of WASH in the retrieval subdomain depends on the cooperative action of different factors. At the same time, WASH activates Arp 2/3 mediated actin polymerization that in turn contributes not only to the stabilization of the retrieval domain but also to the maintenance of WASH endosomal domains [109].

More in general, endosomal actin polymerization could contribute to the stabilization of microdomains in which cargoes are captured, in order to avoid their later diffusion and their entrance in a degradative microdomain (Figure 4). Once a tubular recycling profile is formed, actin contributes not only to its stabilization but also to its fission by providing pushing forces (Figure 4). Apart from the already mentioned roles of WASH dependent actin polymerization, other molecular machineries have been involved in tubule elongation and fission. For instance, the complex BLOC-1 (biogenesis of lysosome-related organelles complex 1) contributes to the elongation and scission of endosomal tubules required for cargo transport to the PM and to melanosomes through interaction with the motor protein KIF13A and AnnexinA2-mediated actin polymerization [110], in a WASH independent manner. A recent work from my host laboratory has shown that the fission of recycling tubules, emanating from mature melanosomes, depends on the motor protein myosin VI and Arp2/3 mediated polymerization of actin in a WASH-dependent manner [111]. A similar process may also occur at endosomes, maybe involving different myosins.

The Class II NPF member cortactin is highly enriched in lamellipodia and membrane ruffles, but it also localized on endosomal and Golgi membranes, where it contributes to post-Golgi transport [112]. Depletion of cortactin causes morphological changes in both endosomes and Golgi and affect the localization and recycling of M6PR [113]. Cortactin directly interact with Arp 2/3 leading to its activation and actin polymerization [114], [115]. Compared to

class I NPF, cortactin is a weak nucleator factor, mostly because it is unable to deliver actin monomer to Arp 2/3 [116]. Cortactin rather interact with the new synthesized branched actin network and contributes to its stabilization [115]. In the context of endosomal recycling, cortactin has been involved in the recycling of  $\beta$ 2AR [117]. Endosomal recruitment of cortactin is mediated by the branched actin network, mostly generated by the WASH complex. Cortactin recruitment and activity is also regulated by PI(3,5)P<sub>2</sub> on endosomal membranes: PI(3,5)P<sub>2</sub> directly binds and sequesters cortactin, preventing its interaction with the actin network and therefore antagonizing cortactin activity [118].

In conclusion, it appears that actin participates in many steps of endosomal functions, including endosomal maturation, capturing of cargoes, stabilization and fission of recycling transport carriers (Figure 4). As described, many of these processes are mediated by the WASH complex and its interaction with the machineries involved in cargo retrieval. In addition, recent studies suggest that the spatiotemporal regulation of actin dynamics depends on the specific composition of endosomal membranes (e.g. PIPs), therefore this aspect needs to be further investigated.



**Figure 4. Role of actin in endosomal trafficking.**

Upon endocytosis, cargoes fated for degradation, such as EGFR, are sorted into ILVs by the ESCRT machinery. Upon full maturation, late endosomes laden with ILVs, fuse with lysosomes to allow cargo degradation. Alongside, ILV formation, cargoes fated for recycling to the PM or for retrograde transport to TGN are sorted into branched tubular profiles, from

where tubulo-vesicular carriers, containing the cargoes, are formed. Actin dynamics contribute to the stabilization of the “retrieval domain” and of the tubular profile, to tubule elongation and fission. In addition, actin polymerization might contribute to short range motility of the tubular carrier. (Adapted from Simonetti & Cullen, 2019)

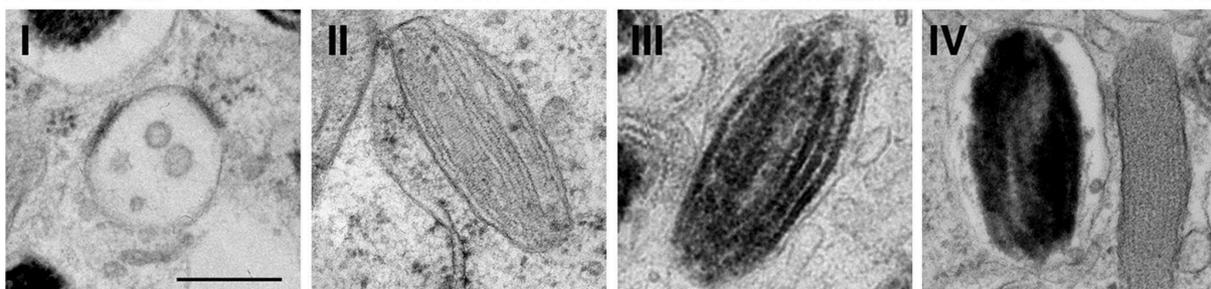
#### 1.4.4. Transport and fusion of the carrier

Once released from the donor compartment, the transport carrier loses its coat and it is transported toward the acceptor compartment. Long-distance transport involves the molecular motors dyneins and kinesins that move along microtubules, while short-range transport is regulated by myosins that move along actin filaments.

The tethering and fusion of the transport carrier with the acceptor compartment is mediated by the family of soluble N-ethylmaleimide-sensitive-factor attachment protein receptors (SNAREs). The fusion is mediated by the interaction of vesicular SNAREs (v-SNAREs) and the target membrane SNAREs (t-SNAREs) to form a tetrameric trans-SNARE complex that allows the apposition and fusion of the two membranes [119].

#### 1.5. Endosomal trafficking in melanocytes and melanosome biogenesis

As previously introduced, melanosomes are lysosome-related organelles found in pigmented cells in the skin and in the eye, where the melanin pigment is synthesized and stored. In skin melanocytes, melanosomes mature through four stages that can be morphologically identified (**Figure 5**). The first two stages (Stage I and Stage II melanosomes, also indicated as premelanosomes or early melanosomes) do not contain melanin but contain amyloid fibrils of the pre-melanosomal protein (PMEL). While PMEL fibril formation starts in Stage I and it is completed in Stage II melanosomes, melanin synthesis only occurs in Stage III melanosomes. Once synthesized, melanin is deposited on the fibrils. For this reason, fully mature Stage IV melanosomes appear as black organelles in which all internal structures are covered by the melanin. In addition to PMEL fibril formation and melanin synthesis, many intracellular trafficking pathways contribute to melanosome maturation and will be briefly described here.



**Figure 5. Ultrastructure of melanosomes.**

Images of Stage I, II, III and IV melanosomes in MNT-1 cells. Stage I melanosomes present a flat clathrin coat. Stage II contain PMEL fibrils and have an ellipsoidal shape. Stage III contain melanin deposits and Stage IV is fully pigmented. Scale bar 200 nm. (from Hurbain et al, 2008)

### 1.5.1. Early melanosomes

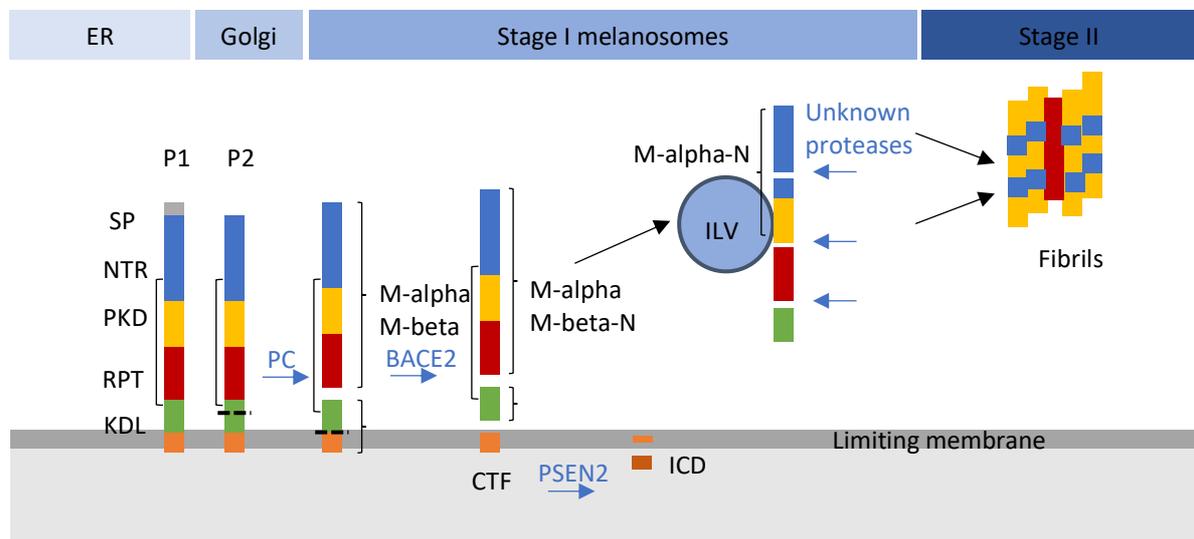
Stage I melanosomes appear as vesicular structures containing ILVs and a clathrin coat, similarly to early endosomes/MVEs. Indeed, Stage I melanosomes are early endosomes, given they are also positive for EEA1 and are reached by endocytic tracers (e.g. BSA) within five minutes [41]. In addition, recycling pathways originating from these early endosomes participate in the transport of cargoes to mature melanosomes.

Apart from PMEL fibril formation (described below), other processes contribute to early melanosomes homeostasis. The ocular albinism type 1 protein (OA1) is a pigment cell specific G-protein-coupled receptor (GPCR) that localizes to melanosomes and lysosomes [120]. Mutations of OA1 cause ocular albinism type I, a defect of eye pigmentation. Depletion of OA1 in melanocytes leads to decreased pigmentation and accumulation of enlarged premelanosomes containing mature melanosome and lysosome proteins [121]. OA1 is sorted to ILVs in both non-melanocytic and melanocytic cell lines in an ubiquitination and ESCRT-dependent manner [122] for its degradation. In addition, OA1 interacts with melanoma antigen recognized by T-cells-1 (MART-1, also called Melan-A), this interaction impacts OA1 stability and degradation. MART-1 also interacts with PMEL and regulates its stability and processing [123], despite its precise role has not been identified yet. A recent work from the host laboratory showed the role of the PIKfyve complex in the maintenance of early endosomes homeostasis and PMEL fibril formation [124]. More in detail, PIKfyve mediates the interaction of early melanosomes with lysosomes, likely for the delivery of proteases required for PMEL processing, and promotes the formation of membrane tubules that contribute to the maintenance of the size and morphology of early melanosomes. Finally, it was recently demonstrated that the small GTPase Rab4A, by interacting with different effectors and likely by creating specific subdomains on the delimiting membrane of Stage I melanosomes, contributes to cargo segregation and to the maturation of Stage I into Stage II melanosomes [125, p. 4].

### 1.5.2. Amyloid fibril formation in early melanosomes

Pre-melanosomal protein PMEL, also called Pmel17, gp100 or Silver in mice, is a major component of melanosomes that through the formation of a “fibrillar matrix” (Figure 6) contributes to melanosome shape and to the optimal polymerization and storage of the melanin. The fibrils contribute also to minimize the diffusion of toxic melanin precursors out of the melanosome [126]. PMEL forms physiological amyloid fibrils, that share features with pathological amyloids formed in the context of neurodegenerative diseases such as Alzheimer’s and Parkinson’s. Although PMEL fibrils are physiological and non-toxic, mutations in PMEL induce the formation of pathogenic fibrils [127]. PMEL is a type I transmembrane glycoprotein containing a short signal peptide (SP) that is co-translationally removed by peptidases, a long luminal N-terminal domain, a single transmembrane domain and a short cytoplasmic C-terminal domain. The luminal domain is formed by four subdomains: named NTR (N-terminal region), PKD (polycystic kidney disease domain), RPT (repeat domain) and KLD (kringle-like domain) [128]. After being synthesized in the ER and

undergoing glycosylation both in the ER and the Golgi [129], [130], it is transported from the TGN to Stage I melanosomes either directly or via the plasma membrane and AP-2 mediated internalization [131]. PMEL undergoes multiple proteolytic cleavage by different proteases, some of which are still unknown. First, full length PMEL is cleaved by a proprotein convertase (PC) in the Golgi apparatus or in a post-Golgi compartment, generating two fragments ( $M\alpha$  and  $M\beta$ ) that remain bound through a disulfide-bound [132], [133].  $M\alpha$  represent the fibrillogenic fragment and comprise the NTR, PKD and RPT subdomains, while  $M\beta$  includes the KLD, the transmembrane domain and the cytosolic domain. Once in Stage I melanosomes, the  $\beta$ -secretase BACE2 ( $\beta$ -site APP cleaving enzyme 2) cleaves PMEL within the  $M\beta$  fragment [134], giving rise to the  $M\beta N$  fragment (that remains associated to  $M\alpha$ ) and to a C-terminal fragment (CTF). At this point, the  $M\alpha$  fragment is further cleaved by other proteases that are still unknown except for ADAM17 [135], while the CTF is furtherly cleaved by a  $\gamma$ -secretase complex containing presenilin 2 (PSEN2) [136].



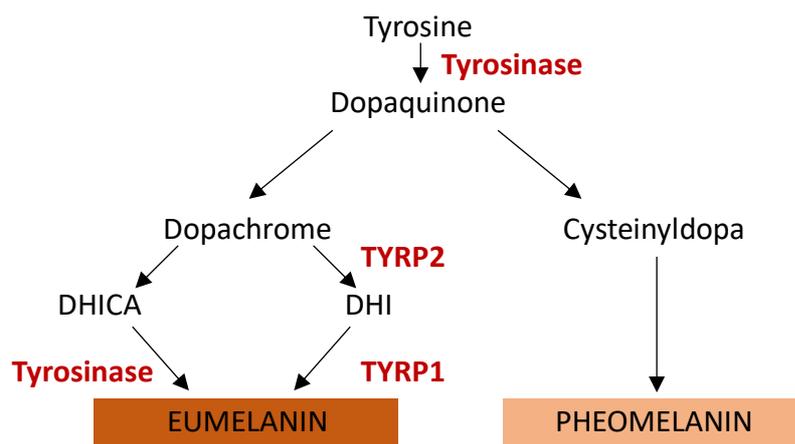
**Figure 6. Schematic representation of PMEL fibril formation.** After cleavage by proprotein convertase (PC) and BACE2, the luminal domain is sorted onto intraluminal vesicles while the C-terminal fragment is fated for degradation in lysosomes. PMEL-processing enzymes are indicated in blue.

Concomitantly with the processing, the generated fragments follow different sorting pathways, with the  $M\alpha$  fragment sorted onto ILVs and the CTF remaining on the delimiting membrane and fated for lysosomal degradation. Indeed, fibril formation occurs inside the melanosome where fibrils radiate from the luminal side of ILVs [137]. Previous works from the host laboratory and collaborators showed that the loading of PMEL  $M\alpha$  on ILVs do not require the cytosolic domain of PMEL, ubiquitination or the ESCRT complex [138], [139]. Contrarily, the sorting of  $M\alpha$  is regulated by the tetraspanin CD63 in an ESCRT-independent manner. Conversely, the CTF is sequestered in the clathrin coat at the limiting membrane in an ESCRT-dependent manner. Depletion of CD63 impact fibril formation and therefore melanosome biogenesis. Interestingly, depletion of CD63 do not affect PMEL processing but

induce the targeting of full-length PMEL into the clathrin coat in an ESCRT dependent manner for degradation. In addition, CD63 interact with PMEL CTF likely maintaining it (and PMEL full-length) in tetraspanin enriched microdomains at the delimiting membrane of the melanosomes. This segregation may contribute to the correct processing of PMEL (mainly by BACE2) and sorting into ILVs. A following study from the host laboratory, in which I participated, illustrates the role of the apolipoprotein E (ApoE) in the loading of PMEL into ILVs and in PMEL fibril formation [140]. This work is further discussed in the **Results, section 1**. Briefly, we showed that ApoE associates with ILVs and interact with the luminal domain of PMEL, allowing the loading of PMEL onto ILVs and correct fibril formation. In addition, we showed that ApoE is involved in the ESCRT-independent pathway regulated by CD63. One of the aims of my PhD was to elucidate the interrelationship between CD63 and ApoE in melanoma cells, hence these data are discussed in more detail in the **Results, section 2**.

### 1.5.3. Mature melanosomes

Mature melanosomes are characterized by melanin deposit onto PMEL fibrillar sheets, hence appearing as black organelles where internal structures are completely hidden. Melanins are a group of pigments found in pigmented cells that originates from oxidation and polymerization of tyrosine. Two main types of melanin can be distinguished: the brown/black insoluble polymer eumelanin and the yellow/red soluble polymer pheomelanin. Both eumelanin and pheomelanin are synthesized in human skin in different ratio, contributing to the variety of pigmentation phenotypes observed in nature. While pheomelanin synthesis mostly requires the enzyme tyrosinase, the synthesis of eumelanin requires additional chemical reactions regulated by the tyrosinase-related proteins TYRP1 and TYRP2 (also called dopachrome tautomerase, DCT) [141] (**Figure 7**).

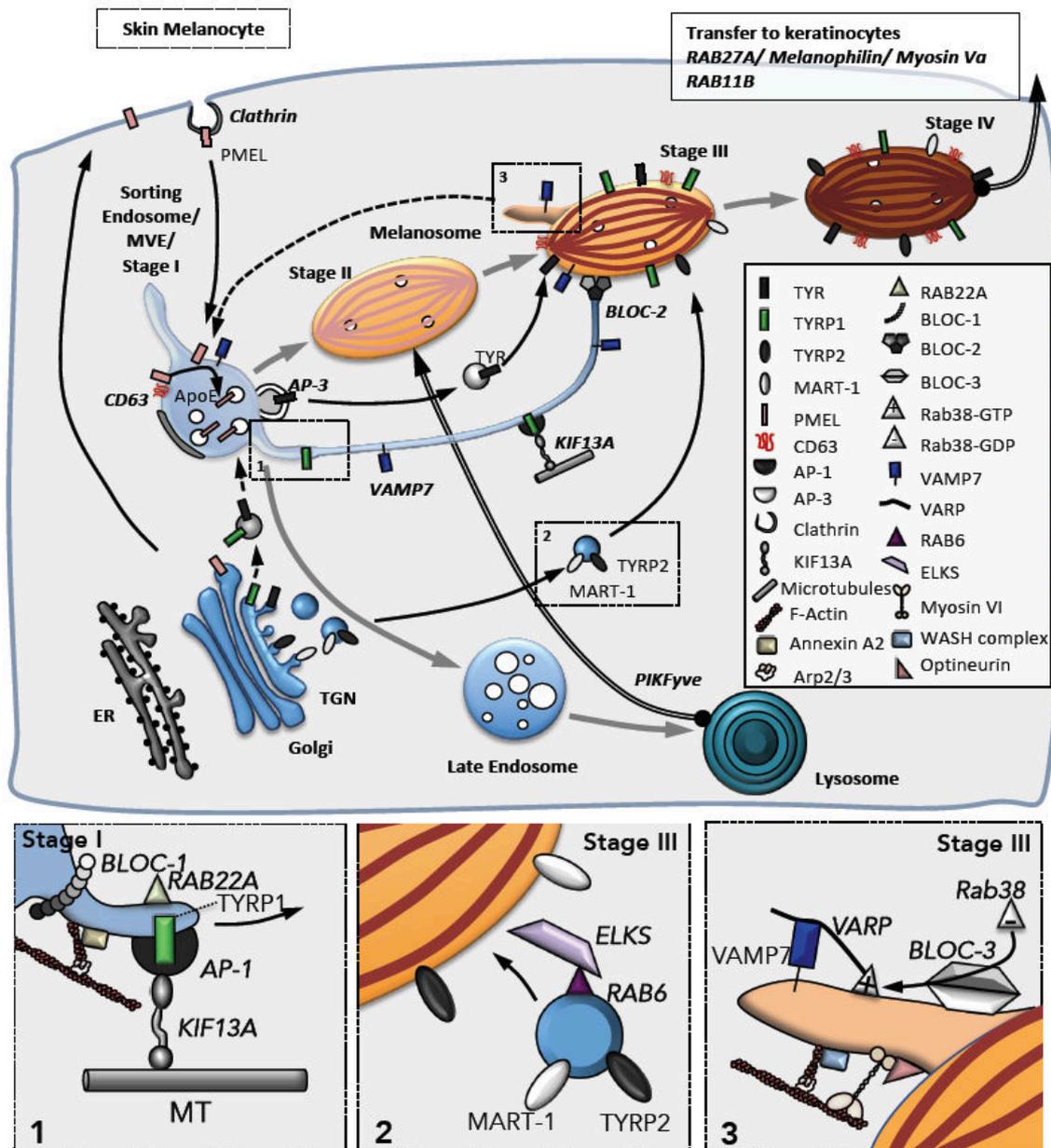


**Figure 7. Biosynthesis of melanin.**  
Enzymes are indicated in red.

Hence, a fundamental process for melanosome maturation is the transport of melanogenic enzymes to maturing melanosomes in order to allow melanin synthesis (**Figure 8**).

Tyrosinase contain a dileucine sorting motif that is recognized by the adaptor protein AP-3. Indeed, tyrosinase and AP-3 colocalize on clathrin-coated buds emanating from early endosomes [142], suggesting that AP-3 mediates the transport of tyrosinase from early endosomes to mature melanosomes. Moreover, tyrosinase is recognized by AP-1 that may mediate an additional transport pathway independently of AP-3. On the contrary, the sorting of TYRP1 depends on AP-1 [13] but not on AP-3 [143]. In particular AP-1, together with the kinesin motor KIF13A mediates the formation of recycling endosomal tubules that establish contacts with melanosomes to deliver TYRP1. Hence, while the trafficking of tyrosinase is mediated by vesicular transport, the trafficking of TYRP-1 is mediated by tubular carriers. Finally, TYRP-2 (together with MART-1) was recently shown to follow a direct pathway TGN- mature melanosomes mediated by the small GTPase Rab6 [144], a protein mostly involved in post-Golgi trafficking to the cell surface. This study shows how specialized cell types can exploit the secretory pathway and divert it towards melanosomes to fulfill their specific functions.

In addition to adaptor proteins, the biogenesis of LROs is regulated by the biogenesis of lysosome-related organelles complexes BLOC-1, BLOC-2 and BLOC-3 (**Figure 8**). BLOC-1 participates to the trafficking of several proteins from early endosomes to mature melanosomes, such as the copper transporter ATP7A [145], the oculocutaneous albinism II OCA2 [146] and TYRP1 [147] through the formation of recycling tubules. BLOC-1 also cooperates with the cytoskeleton to allow the formation of recycling tubules in both non-pigmented and pigmented cells, where it sustains melanosome maturation [110]. Hence, BLOC-1 may be involved in the same pathway of AP-1 for the transport of TYRP1 but the exact mechanism needs to be further elucidated. Interestingly, BLOC-1 also interact with AP-3 acting together in the trafficking of OCA2 [146]–[148]. BLOC-2 localizes in recycling tubules functioning in the same pathway of BLOC-1 where it has a role in directing endosomal recycling tubules towards melanosomes and stabilizing their contact with melanosomes [149]. BLOC-3 function as a guanine nucleotide exchange factor for the GTPase Rab32/38 [150, p. 3] and seems to play a role in the exit of cargoes from mature melanosomes [151]. In particular, while BLOC-1 contributes to the anterograde transport of the v-SNARE VAMP7 to mature melanosomes, BLOC-3 regulates its exit from mature melanosomes together with its scaffold protein VARP. The formation and release of VAMP7 positive recycling tubules is mediated by myosin VI and the actin cytoskeleton [111], suggesting that BLOC-3 and myosin VI are involved in the same pathway, nevertheless if BLOC-3 and Rab32/38 are required for myosin VI recruitment is still not known. Finally, other proteins involved in cargo trafficking to mature melanosomes include Rab proteins as Rab9 [152] and Rab32/38 [153, p. 38] and SNARE proteins such as syntaxin13 [154].



**Figure 8. Intracellular trafficking pathways contributing to melanosome biogenesis.**

Trafficking pathways from the Golgi apparatus and early endosomes to generate different stages of melanosomes are shown. Following endocytosis, PMEL is transported to stage I melanosomes where fibril formation, regulated by CD63 and ApoE, begins. Full maturation into Stage II melanosomes requires interaction with lysosomes, mediated by PIKFyve, and the organization of the fibrils into sheets. Melanogenic enzymes are transported to Stage III melanosomes through several mechanisms. Transport of Tyrp1 and VAMP7 requires the formation of recycling tubules mediated by BLOC-1 and AP-1 (box 1). BLOC-2 contribute to the targeting of these tubules to maturing Stage III melanosomes. Transport of Tyrosinase is mediated by AP-3 vesicles. Tyrp2 and MART-1 are transported directly from the Golgi through Rab6 positive vesicles (box2). VAMP7 is retrieved from Stage III melanosomes through recycling tubules that require myosin VI and actin for their constriction and scission (box 3). Mature Stage IV melanosomes are transported at the periphery of skin melanocytes, captured by the Rab27A/Melanophilin/myosin Va complex and transferred to keratinocytes, likely after fusion mediated by Rab11b. (adapted from Delevoye et al, 2019).

#### 1.5.4. Transport and fate of melanosomes

As melanosomes mature, they are transported from the perinuclear region to the periphery of the cells in particular to dendrites, plasma membrane extension of skin melanocytes that can contact up to 40 keratinocytes, where the transfer of melanosomes to keratinocytes is supposed to occur. Melanosome transport rely on microtubules for long-distance transport up to the cell surface where melanosomes are captured by the cortical actin cytoskeleton. The docking of melanosomes to the cortical actin cytoskeleton require a tripartite complex formed by Rab27a, melanophilin and myosin V [155].

Once at the cell periphery, mature melanosomes need to be transferred to keratinocytes to contribute to skin pigmentation and photoprotection. Although different models of melanosome transfer have been proposed [156], new evidences suggest that the transfer occurs through a process of exocytosis from melanocytes and endocytosis by keratinocytes. Such process implies the fusion of melanosome membrane with the plasma membrane, likely mediated by Rab11 [157], the release of melanosome content called melanocore in the extracellular space and its following uptake by keratinocytes, that requires the G-protein coupled receptor PAR-2 [158]. Once in keratinocytes, melanocores are stored in membrane-bound organelles positive for lysosomal membrane proteins, non-degradative and non-acidic [158]. These organelles were also found in human skin biopsies, where the distribution of melanocores in keratinocytes was also dependent on the skin phototype [159].

In conclusion, melanocytes are specialized cells in which intracellular trafficking pathways have been in part adapted to fulfill specific functions. Moreover, the formation of physiological PMEL amyloids share features with the formation of pathological amyloids. For these reasons, melanocytes represent a good model not only for the study of intracellular trafficking pathways, but also for the study of amyloid fibril formation, which can contribute to a better understanding of neurodegenerative diseases.

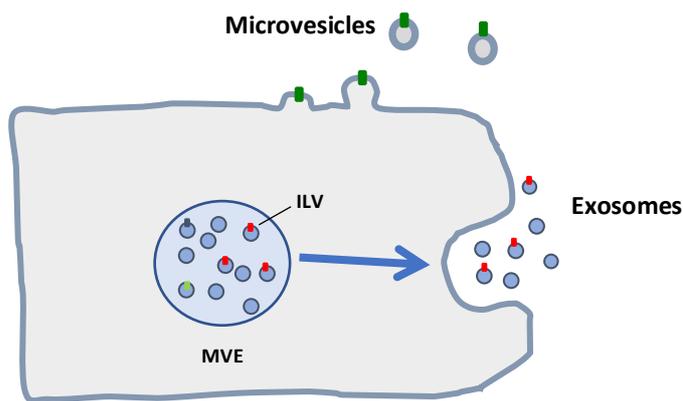
The endosomal system is a dynamic and interconnected network of compartments. By playing a central role in the transport, sorting and degradation of proteins and lipids, the endosomal system contribute to fundamental cellular processes and to the maintenance of cellular homeostasis.

## 2. EVs and exosomes

### 2.1. EVs subtypes, nomenclature and standardization of EV research

Extracellular vesicles (EVs) are small vesicles delimited by a lipid bilayer and released in the extracellular environment by all kind of cells and organisms. Despite EVs were first described as a mean for the elimination of unneeded components from the cell, it is now clear that these vesicles can be taken up by nearby or distant cells and modulate their functions. Hence, EVs act as intercellular communicator, in addition to secreted factors (e.g. cytokines) and direct cell-cell contact.

The term “extracellular vesicles” generally describes all secreted membrane vesicles, independently from their biological/physical properties and their origin. Indeed, inside the heterogenous group of EVs different classifications can be done. First, EVs can be classified according to their origin. In these case two main classes can be distinguished: EVs of endosomal origin, also indicated as exosomes, and EVs derived from the plasma membrane, also indicated as microvesicles (or ectosomes) (Figure 9).



**Figure 9. Classes of extracellular vesicles.**

Extracellular vesicles are formed either by budding of the plasma membrane, in which case they are referred to as microvesicles, or as intraluminal vesicles (ILVs) within the lumen of multivesicular endosomes (MVEs). Upon fusion with the plasma membrane, MVEs release ILVs that are then called exosomes.

Exosomes are released upon the fusion of a multivesicular endosome with the plasma membrane, hence they correspond to ILVs formed in this compartment. On the contrary, microvesicles originate through outward budding and fission of the PM. Exosomes are usually 30-200nm in diameter, while microvesicles were originally described as larger (200 nm-1000 nm), but their size actually ranges from 50 nm to 1000 nm, up to 10  $\mu$ m in the case of large oncosomes, a class of microvesicles released by cancer cells [160]. In addition, migrating cells can release large vesicles, containing numerous smaller vesicles, that grow on the tips and intersections of retraction fibers at the rear of migrating cells and are finally released [161]. Such vesicles are called migrasomes and are enriched in tetraspanin TSPAN4 and the integrins  $\alpha$ 5 and  $\beta$ 1 [162]. The microvesicle family also include the arrestin-domain-containing protein1 (ARRDC1) -mediated microvesicles (ARMMs), 50 nm vesicles originated by outward budding of the PM through a mechanism that requires ARRDC1 and the endosomal sorting complex required for transport (ESCRT) (similarly to exosomes, see

below) [163]. ARMMs contain active NOTCH receptors and can mediate NOTCH signaling in recipient cells [164]. In addition, ARRDC1 seem to be also involved in the biogenesis of endosome-derived exosomes [165].

The biogenesis of microvesicles shares some features with the biogenesis of exosomes. First, in both cases the vesicles are budding out from the cytoplasm, contrarily to intracellular transport vesicles originated in other cellular compartments. This also implies that the topology of transmembrane proteins in exosomes and microvesicles is the same than at the PM. Second, and as in the case of transport vesicles, the first step of exosome and microvesicle biogenesis requires the clustering of cargoes to create a microdomain from which the initial bud will originate. At this point the mechanisms start to differ, despite some molecular machineries are partially shared. Some examples are represented by the ESCRT proteins and by sphingomyelinase that mediates the synthesis of ceramide. The study of exosome biogenesis being one of the main foci of my thesis, the mechanisms involved will be extensively described below, while for microvesicles, further details can be found in more specific reviews [166], [167]. Briefly, microvesicle biogenesis requires changes in lipid and protein composition of the PM, of  $Ca^{2+}$  levels and rearrangement of actin and microtubule cytoskeleton. A unique process contributing to microvesicle budding is the flipping of specific lipids from one leaflet of the PM to the other, that contributes to membrane bending [168]. Finally, the budding process requires actomyosin contraction to generate the forces requires for vesicle fission.

In addition to exosomes and microvesicles, other classes of EVs or “EV-related particles” have been recently identified. Use of asymmetric flow field-flow fractionation identified a new class of small particles called exomeres [169]. Exomeres are ~35 nm membranous particles that lack a lipid bilayer but contain lipids and are enriched in microtubule proteins, proteins involved in hypoxia and coagulation and metabolic enzymes. A second study purified exomeres using differential ultracentrifugation and identified several cargoes including Argonaute proteins and amyloid precursor protein (APP). In addition, other two cargoes, the  $\beta$ -galactoside  $\alpha$ 2,6-sialyltransferase 1 (ST6Gal-I) that  $\alpha$ 2,6- sialylates N-glycans and the EGFR ligand amphiregulin (AREG) are functionally transferred to recipient cells and elicit specific functions [170]. Although exomeres seem to have a functional relevance and contain components also found in EVs, they contain lipids, but they don't have a lipid bilayer. Hence, they cannot be considered vesicles. A recent study employed high-resolution density gradient to separate EVs from non-vesicular material identifying non-vesicular fractions, separated from small EVs, enriched in metabolic enzymes and cytosolic proteins (e.g. tubulin) that indeed resemble exomeres [171].

In addition, other EV classifications and denominations found in the literature divide EVs by size as small EVs (less than 100nm or 200nm) and large/medium EVs (more than 200nm), by density (low, middle, high) or biochemical composition (e.g. tetraspanin positive EVs).

In conclusion, considering the high heterogeneity of extracellular vesicles, the term “extracellular vesicles” should be used especially when the biogenesis mechanisms are not

investigated, as indicated by the guidelines recently published by the International Society of Extracellular Vesicles (ISEV) [172].

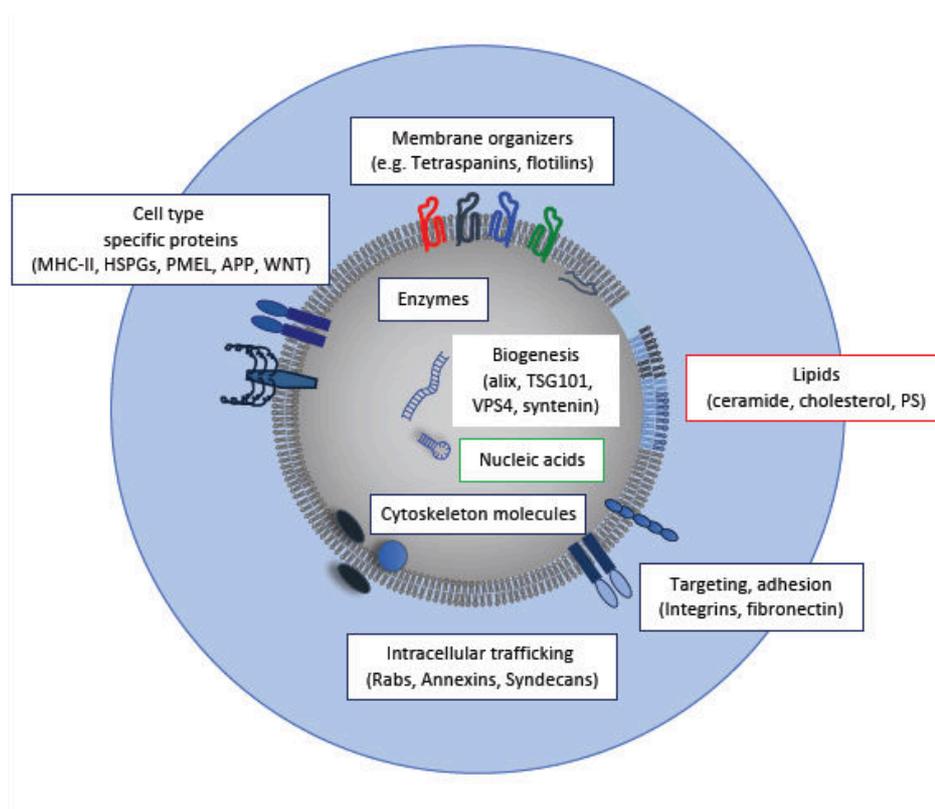
Moreover, research in the field of EVs has rapidly grown in the last decade with an exponential increase in the number of EV-related scientific publications; new technical approaches have been developed for both isolation and characterization of EVs, limitations of some technical approaches have been reported; new EV subtypes or “EV-related particles” have been reported and many new functions have been ascribed to EVs. Hence, considering the complexity and challenges associated with EV research, standardization of EV research and transparent reporting of employed methodology appear of fundamental importance. In this context, I participated to the development of the crowdsourcing knowledgebase EV-TRACK (<http://evtrack.org>) [173] (see **Annexes, section 2**). EV-TRACK is an online toolset that comprise methodological specifications of scientific works published after 2010 and where published and unpublished works in the EV field can be inserted in order to measure transparent reporting of isolation and characterization methods (this score is indicated as EV-METRIC). Overall, this tool aims to help authors to improve the reporting of methodological information in order to facilitate the interpretation and reproducibility of EV-related scientific works. Of note, such guidelines and ISEV guidelines [172] were followed as much as possible in the writing of the **Result section** in this manuscript and the obtained data will be inserted in the EV-TRACK platform.

## **2.2. Properties and composition of EVs**

As aforementioned EVs are an heterogenous group of membrane vesicles in terms of origin, size, density, biochemical and physical properties and composition. Such heterogeneity is reflected by the multitude of functions attributed to EVs.

In addition, the techniques currently used for EV isolation and characterization are based on their specific properties. For instance, differential ultracentrifugation, that has been considered for long time as the standard protocol for EV isolation, separates EVs according to their size, with large EVs pelleting at 10,000 g and small EVs pelleting at 100,000 g. It is now clear that the 100,000g pellet contains also contaminants such as protein aggregates. Hence, techniques such as size exclusion chromatography represent a good alternative to efficiently separate EVs from protein contaminants due to their different size. Alternatively, a separation based on density can be achieved using a density gradient. This technique has allowed the identification of EV subpopulations and their separation from non-vesicular components [171], [174]. The morphology and size of EVs can be easily assessed by electron microscopy (EM) that in addition remain the standard method for the study of EV biogenesis inside the cell. Recent reports using cryo-EM, a technique that allows analysis of EVs in their native state without chemical fixation, revealed not only the spherical morphology of EVs, as expected, but also the presence of unexpected structures and morphologies [140], [175], [176]. Investigation of the size and the number of EVs can also be performed using nanoparticle tracking analysis (NTA) and tunable resistive pulse sensing (TRPS) although these instruments have a detection limit of about 50nm in size and do not discriminate EVs

from other particles and contaminants with similar size. In terms of composition, EVs contain specific sets of proteins, lipids and nucleic acids. Such specificity is dependent on their cell type of origin, on their biogenesis mechanisms but also on the conditions that determined exosome release (e.g. presence of a pathological condition). In this context, Western Blot easily allows the detection of a variety of proteins in EV pellets without distinguishing soluble, membrane associated and luminal proteins. Alternatively, immuno EM can be used but only for epitopes exposed on the surface of EVs. Moreover, the use of high throughput techniques (proteomics, lipidomics, transcriptomics) identified a large amount of EV components. These collections of data can be found in specific databases, such as Vesiclepedia and EVpedia [177], [178]. Of note, studies comparing the content of microvesicles, exosomes and their cell of origin suggest that the composition of microvesicles is more similar to their cell of origin, while exosome composition differs the most. Here an overview of the main protein, lipid and nucleic acid found in EVs is given (Figure 10).



**Figure 10. Overview of EV composition.**

### 2.2.1. Proteins

EVs contain a broad variety of proteins including transmembrane proteins, associated membrane proteins and cytosolic proteins. Among transmembrane proteins, the most abundant are the tetraspanins CD63, CD81 and CD9, that are broadly used as markers of EVs. Despite in the cell CD63 is highly enriched in MVEs while CD81 and CD9 are highly enriched at the PM, CD63 is also found in EVs budding from the PM and CD81 and CD9 are

also found in MVE derived exosomes. Hence, their relative abundance and localization can be cell type dependent. Moreover, the presence of tetraspanin-negative EVs has been reported [179]. Tetraspanins are known for their specific organization in microdomains together with other proteins, such as syntenin, integrins, metalloproteases, adhesion molecules, MHC class II. These proteins can also be found in EVs. Proteins involved in EV biogenesis are clearly found in EVs. These include the ESCRT components ALIX, TSG101 and VPS4 for both exosomes and microvesicles, syntenin and CHMP4A-B for exosomes and ERK and PLD for microvesicles. Other proteins involved in membrane trafficking include Annexins, the lipid-raft component flotillins, EHD4 and Syndecans. Cytoskeletal proteins (e.g. actin, tubulin) and metabolic enzymes (e.g. GAPDH) have been found in EVs, nevertheless they may be associated to non-vesicular contaminants more than to EVs [171]. Similarly, ECM components such as fibronectin have been found in EVs but also in non-vesicular contaminants [171], but such discrepancies may be cell dependent.

Despite any unique and specific marker for a given subpopulation of EVs exists at the moment, some specific markers have been proposed. Kowal et al. proposed the use of tetraspanins together with syntenin and TSG101 to identify “bona fide” exosomes [174]. Recently Jeppesen et al. proposed AnnexinA1 as a specific marker of microvesicles but not of exosomes or ARMMs [171], while Minciacchi et al. proposed cytokeratin 18 as a specific marker of large oncosomes secreted by tumor cells [180].

### **2.2.2. Lipids**

Different lipid species have been identified in EVs, while the biosynthesis of some species seem to be required for EV biogenesis. Examples of lipids identified in exosomes are phosphatidylcholine, phosphatidylserine (PS), phosphatidylethanolamine, phosphatidylinositols, phosphatidic acid, cholesterol, ceramides, sphingomyelin and glycosphingolipids [181]. Cholesterol is highly enriched in ILVs [182], hence it is easily found in exosomes [181], [182]. Ceramide synthesis by sphingomyelinase is essential for the biogenesis of exosomes in a ESCRT-independent manner [183]. Of note, lyso-bisphosphatidic acid (LBPA, also known as bismonoacylglycerol phosphate, BMP), a lipid uniquely present in late endosomes and highly enriched in internal membrane of these compartments [184], is not found in exosomes [181], suggesting that LBPA may be likely present only in ILVs fated for lysosomal degradation.

Differently from exosomes, microvesicles seem to be more enriched in phosphatidylserine, phosphatidylethanolamine and sphingolipids.

Lipid composition of EVs appear also different from lipid composition in the cell of origin, with some species being highly enriched in EVs and other being highly enriched in the cell. In addition, the distribution of lipids between the inner leaflet and the outer leaflet of EV membranes may be different [181]. One interesting case is represented by phosphatidylserine. PS is normally localized in the inner leaflet of membranes, but it can be exposed on the outer leaflet when cells undergo apoptosis, representing an “eat-me signal”. PS is described to be present also in the outer leaflet of EV membranes, as it can be detected

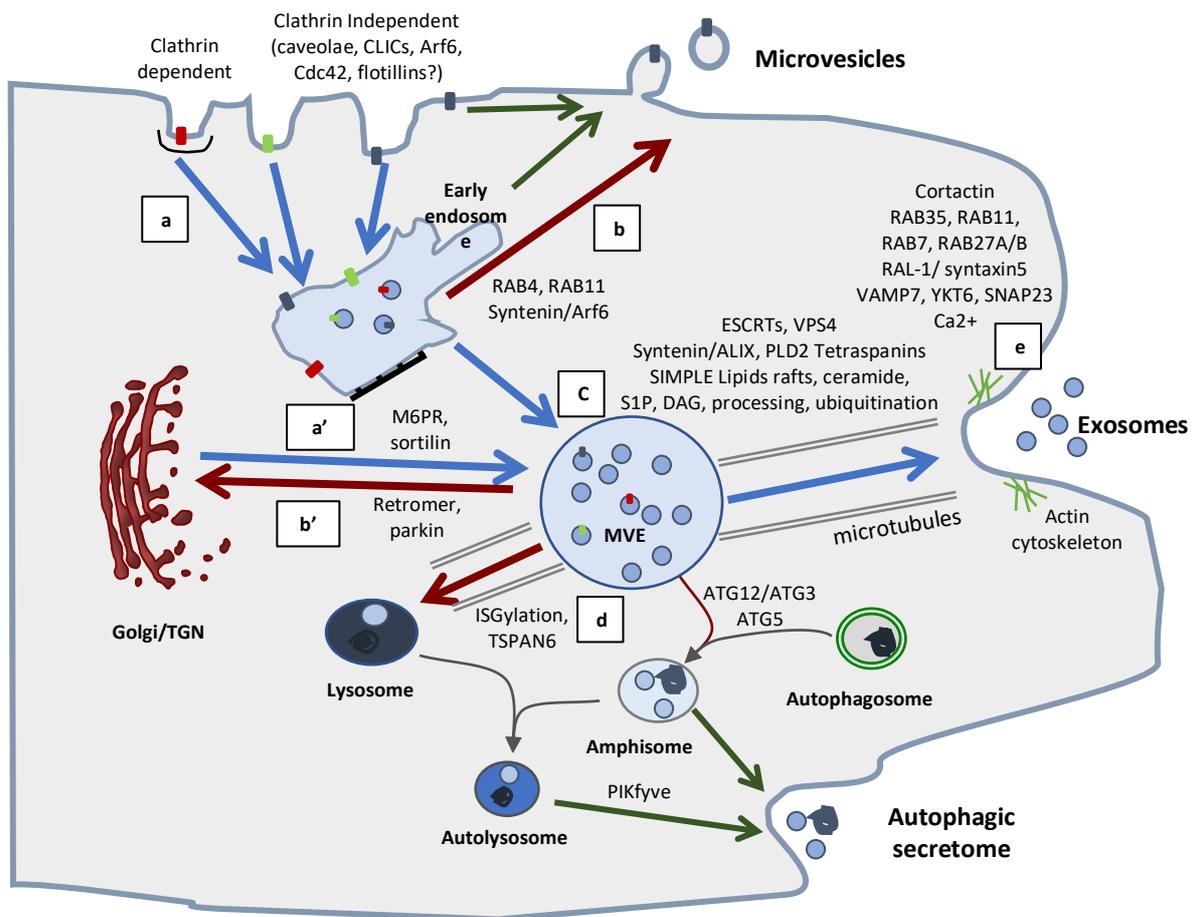
using AnnexinA5 or TIM-4. While it is still not clear if PS exposure take place after vesicle release, it may be a feature of EVs released under particular conditions, for instance hypoxia and may contribute to their uptake by recipient cells [185]. Although PS exposure may occur also in non-apoptotic, healthy cells, it should not be considered as a general feature of EVs.

### **2.2.3. Nucleic acids**

Nucleic acids found in EVs include DNA, mRNA, microRNAs and other non-coding RNAs [186], [187]. Earlier studies described that mRNAs and miRNAs present in exosomes can be functionally transferred and be expressed in target cells [188]–[191], despite the amount of miRNA per vesicles may be very low [192]. The sorting of RNAs in EVs may occur through a passive loading or active loading mediated by RNA-binding proteins (RBPs), such as Ago2 [193] or the major vault protein [194] or the Y-box protein 1 [195]. Nevertheless, the presence of RBPs in EVs remains controversial. Ago proteins and Vault proteins were found in non-vesicular components [171] and they can form RNA-containing particles found in human plasma [196]. The study from Jeppesen et al. also observed the absence of cytosolic DNA in EVs and described a new pathway of secretion for DNA that depends on autophagy. In particular they propose that cytosolic DNA and histones are secreted upon the fusion of an amphisome, the product of the fusion of an autophagosome with an MVE, with the plasma membrane. Of note, amphisomes can also contain ILVs that can be released as exosomes, but they are not associated to DNA.

### **2.3. Mechanisms of ILV biogenesis**

As aforementioned, ILV formation is a fundamental process contributing to endosome maturation. ILV formation in MVE is important for both cargoes fated for secretion on exosomes and for cargoes fated for lysosomal degradation. Different pathways have been shown to contribute to cargo sorting into future ILVs and to ILV formation and budding. Considering the high complexity and dynamicity of the endosomal system, MVE appear to be at the crossroad of several intracellular pathways that can determine both the fate of a cargo and the fate of an MVE (**Figure 11**). Hence, different mechanisms, including the intracellular trafficking of cargo proteins to MVEs, the sorting of cargoes into ILVs and the targeting of MVEs to the PM, can be considered as regulatory elements of exosome biogenesis. These mechanisms will be described in this section and were also illustrated in a review I wrote in 2018 and that can be found in **Annexes, section 1**.

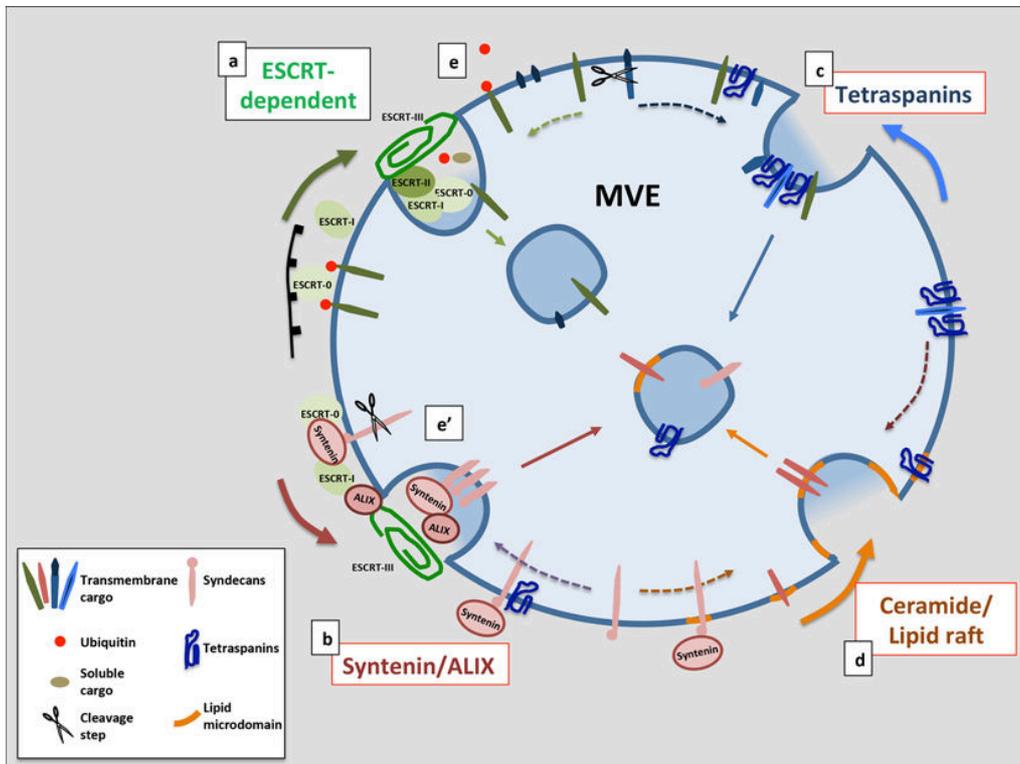


**Figure 11. Intracellular trafficking checkpoints involved in exosome biogenesis and secretion.**

The letters in white squares indicate the different checkpoints. The blue arrows highlight the trafficking steps favoring exosome biogenesis and the red arrows highlight the trafficking steps impairing exosome biogenesis. The green arrows represent alternative release mechanisms (microvesicles and autophagic secretion). Exosome biogenesis and release are regulated by multiple checkpoints that are annotated by the letters ‘a’ to ‘e’. Exosomal cargo proteins reach the endosomal system (a) through endocytosis or (a’) by direct transport from the Golgi apparatus. Before being sorted into ILVs, the cargo proteins need to avoid recycling to (b) the PM or (b’) the Golgi apparatus. (c) Once formed, MVEs have to escape different fates such as fusion with (d) lysosomes or (d) autophagosomes and be transported towards the PM in order (e) to fuse with it and secrete exosomes into the extracellular environment. The main reported regulators of each step are indicated. (from Palmulli & van Niel, 2018).

### 2.3.1. ESCRT- dependent and -independent pathways

As described earlier in this manuscript, the best characterized mechanism of ILV biogenesis requires the ESCRT complex (Figure 12).



**Figure 12. Sorting mechanisms at MVEs involved in exosome biogenesis**

The bold arrows outside the MVEs indicate the main sorting mechanisms reported so far. The dashed arrows within the lumen of the MVEs indicate a potential intersection or coordination between sorting mechanisms. Four main sorting mechanisms have been reported so far for transmembrane proteins: (a) the classic ESCRT mechanism, (b) the syntenin–ALIX pathway, (c) the tetraspanin microdomains and (d) the ceramide pathway. (e, e') Post-translational modifications are key regulators, allowing controlled targeting of a modified cargo protein (ubiquitination, cleavage) to a specific sorting mechanism. (from Palmulli & van Niel, 2018).

As mentioned, the last step of ESCRT- dependent ILV formation consists in the recruitment of deubiquitinating enzymes that remove the ubiquitin tag from cargo proteins [33], [34] before that the newly formed ILV is released into the lumen of MVEs. However, ubiquitinated proteins can be still found in exosomes, suggesting that deubiquitination is not a critical step in exosome biogenesis [197]. Several studies have shown the participation of ESCRT-dependent mechanism to exosome biogenesis. Colombo et al. performed an RNA interference screening targeting 23 ESCRT and ESCRT-associated proteins in HeLa cells to evaluate the role of the ESCRT complex in exosome biogenesis [198], identifying 7 proteins that affect exosome secretion. Although depletion of ESCRT-0 or -I components STAM, HRS

or TSG101 reduced exosome secretion, depletion of ESCRT-III CHMP4C, VPS4 and accessory molecules VTA1 or ALIX increased it. In the same study, depletion of ALIX increased MHC-II secretion on exosomes but had variable effects on CD63 and HSC70 levels, suggesting that ALIX may regulate more the composition of exosomes than global exosomal secretion. Of note, this study mainly analyzed exosomes released from HeLa cells expressing the CIITA transactivator, hence, it is still unclear which steps of exosome biogenesis were affected and whether they are specific to the forced expression of MHC-II. Recently, inhibition of VPS4 in HEK293 cells has been shown to decrease release of different subpopulations of EVs, corresponding to CD63-enriched exosomes and CD9-enriched microvesicles [199]. In addition, the ESCRT-0 protein HRS seem to regulate exosome secretion in dendritic cells [200], the release of exosomal Wnt3 [201], and its depletion was shown to decrease overall exosome number, as measured by nanoparticle tracking analysis [202]. Interestingly, electron microscopy has shown that HeLa cells depleted for HRS, display MVEs containing small ILVs (<40 nm in diameter) [203]. Therefore, it cannot be excluded that some subpopulations of exosomes, due to their small size, are below the detection threshold of some investigative methods (such as NTA) or still lack specific markers for their detection by Western Blot.

The ESCRT mechanism can be partially or completely dispensable for the generation of ILVs. This is the case of the syndecan-syntenin-ALIX pathway (**Figure 12**). Syndecans are transmembrane heparan sulfate proteoglycans that can interact with the PDZ domain containing protein syntenin [204]. Syntenin also interact with ALIX. Together, syndecans, syntenin and ALIX assemble in a tripartite complex which can drive not only their own exosomal release, but also the release of CD63 and HSP70 [205]. This pathway is also regulated by ADP-ribosylation factor 6 (Arf6) and its effector Phospholipase D (PLD2) [206]. In order to identify ESCRT components involved in syndecan–syntenin–ALIX exosome biogenesis, Baietti et al. [205] performed an RNA interference screening on MCF-7 cells. Depletion of TSG101 (ESCRT-I), VPS22 (ESCRT-II), CHMP4 (ESCRT-III) or VPS4 reduced exosomal release of syndecans, syntenin and CD63, suggesting that these components are involved in this pathway. However, in contrast to the conventional ESCRT pathway, ALIX may recruit directly CHMP4 and VPS4. It should be noted that this study mostly analyzed the specific syndecan–syntenin exosome subpopulation in MCF-7 which would explain the discrepancies with other reports investigating similar processes in HeLa cell CIITA [198]. Hence, the preferential mechanism for biogenesis can vary depending on the cell type and the cargo proteins enriched in specific subpopulations of exosomes. Finally, a recent study from my host laboratory showed the importance of syntenin in exosome biogenesis and function in vivo, using CD63-phluorin as a reporter of exosome secretion in zebrafish [207]. ESCRT-independent biogenesis of ILVs (**Figure 12**) was first revealed using simultaneous depletion of components of the four ESCRT subcomplexes [208]. Electron microscopic analyses have shown ILVs formed in absence of ESCRT components are enriched in the tetraspanin CD63 [203], [208]. As described above, tetraspanin are enriched in exosomes and are widely used as exosome markers. Indeed, different tetraspanins have been

proposed to have a role in ILV formation. Bone marrow dendritic cells from CD9 knockout mice secrete fewer exosomes compared with wild-type mice, whereas expression of CD9 and CD82 promotes the release of  $\beta$ -catenin in exosomes [209]. In a mouse model of breast cancer, the release of Wnt11 on exosomes from cancer-associated fibroblasts depends on the tetraspanin CD81 [210]. Expression of another tetraspanin, Tspan8, has been shown to change exosomal protein and mRNA content in rat adenocarcinoma cells [211] and to increase release of EVs containing E-cadherin and p120-catenin in the circulation of tumor-bearing mice [212, p. 8]. Finally, the tetraspanin CD63 plays an important role in exosome biogenesis in multiple cell types. CD63 is required for the generation of small ILVs in HeLa cells depleted for HRS [203] and has recently been involved in the biogenesis of exosomes in fibroblasts from patients with Down's syndrome [213]. CD63 also targets the Epstein–Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) to ILVs and it is critical for its secretion on exosomes [214], [215]. As described above, in melanocytes, CD63 is required for the sorting of the melanosomal protein PMEL to ILVs [139], that also requires the targeting of ApoE to ILVs and exosomes [140] but not the ESCRT complex [139].

In addition to proteins, lipids are also involved in exosome biogenesis (Figure 12). Indeed, the first ESCRT-independent pathway was reported in Oli-neu cells and it requires generation of the sphingolipid ceramide via hydrolysis of sphingomyelin by neutral type II sphingomyelinase [183]. Ceramide could create specific lipid microdomains and induce negative membrane curvature, which give rise to ILVs and exosomes enriched in ceramide. In addition a metabolite of ceramide, sphingosine 1-phosphate, has recently been shown to continuously activate Gi-protein-coupled sphingosine 1-phosphate receptor and in this way to regulate the sorting of cargo proteins into exosomal ILVs [216]. Nevertheless, its precise role in endosomes and ILV biogenesis needs to be further elucidated. Moreover, lipid rafts, which are specific lipid microdomains enriched with cholesterol and glycosphingolipids can contribute to ILV formation and sorting of raft-associated molecules such as GPI-anchored proteins [217] or of annexin A2 [218]. Inhibition of DGK, an enzyme that metabolizes Diacylglycerol (DAG) into phosphatidic acid, has been shown to induce release of exosomes in T-cells [219]. Finally, LBPA is specifically enriched in late endosomes such as MVEs and can recruit ALIX to generate ILVs in vitro and in vivo [220], [221]. Nevertheless, LBPA is not abundant in exosomes [181], hence, LBPA may be required more for the biogenesis of ILVs fated for lysosomal degradation. Finally the syntenin–ALIX pathway is also regulated by PLD2 which hydrolyses phosphatidylcholine to generate choline and phosphatidic acid [206].

Overall, it appears that cargo sorting at MVEs involves several mechanisms that are not necessarily mutually exclusive. As described above, membrane curvature is determined by both lipid composition and membrane protein oligomerization and shape, hence it is likely that proteins and lipids cooperate in order to induce the budding of specific microdomains. A potential example is given by a recent structural study of the tetraspanin CD81, which revealed a cone-like structure with an intramembrane pocket that can bind cholesterol [222]. In this case one can imagine that the clustering of several cone-shaped tetraspanins

could then induce inward budding of these microdomains. Another interrelationship between sorting mechanisms is provided by the known interaction of CD63 with syntenin [223] or the regulation of CD63 by the ALIX–syntenin pathway [205]. Moreover, ceramide regulates the syntenin–ALIX pathway in MCF cells [205] and the sorting of CD63 in Oli-neu cells and in HeLa cells [183], [224]. Interconnection between different pathways may also be cell type specific, as exemplified by the sorting of PMEL through ApoE, that interestingly has also particular affinity for lipids, which depends on CD63 but not on ESCRT or ceramide [139], [140] in pigment cells. In this model, ESCRT-dependent and -independent mechanisms for ILV biogenesis are tightly coordinated on the same MVE by the tetraspanin CD63 [139]. The sorting of non-ubiquitinated cargo proteins can also require both ESCRT-dependent and -independent mechanisms. In fact, these cargoes can be trapped in microdomains formed by the clustering of the highly ubiquitinated tetraspanin protein Cos. This ubiquitination ‘in trans’ allows the recruitment of the ESCRT mechanism and ‘canonical’ formation of ILVs [225].

However, it is unlikely that all sorting mechanisms act on the formation of a single ILV, as supported by the observation that ESCRT-dependent and -independent mechanisms give rise to a heterogeneous population of ILVs in terms of both size and cargo proteins [203].

Overall, the variety of sorting mechanisms in a single cell type will determine the heterogeneity of ILVs that the cells will generate among which a proportion will be released as exosomes. As mentioned, ILV formation is also required for lysosomal cargo degradation. Despite the relative contribution of each single pathway to exosome secretion or lysosomal degradation is still not known, it seems that ESCRT-independent and semi-dependent mechanisms (such as the syntenin–ALIX pathway) would favor ILV release as exosomes while the conventional ESCRT-dependent mechanisms may be more dedicated to the degradation pathway.

### **2.3.2. Other mechanisms influencing exosome biogenesis**

In addition to the formation of ILVs per se, exosome biogenesis is influenced by several other mechanisms, including the expression of a given cargo and the modifications it may undergo.

Expression of cargo proteins destined for exosomal secretion can be viewed as the first regulator of exosome formation because the targeting of a particular protein cargo to MVEs can induce the recruitment of specific machineries leading to ILV formation. This is exemplified by the observation that ectopic expression of MHC class II in HeLa cells [226], or its forced expression in intestinal epithelial cells after stimulation by interferon  $\gamma$  (IFN- $\gamma$ ) or expression of its transactivator CIITA [227], increased the release of EVs by promoting the recruitment of the sorting machineries for ILV generation. Expression of the melanosomal protein PMEL in HeLa cells induces not only its sorting to ILVs in an ESCRT-independent manner [138], but also the generation of subpopulations of ILVs [203], [228] and the production of exosomes. Similarly, overexpression and depletion of syndecans from MCF-7 cells increase and decrease the recovery of exosomal markers respectively [205]. Overall, the

expression of a cargo protein, which is either cell type specific or induced by external stimuli, appear as the first regulatory element influencing exosome biogenesis and composition. Hence, the cellular expression of a given cargo or the formation of ILVs should be investigated in parallel to exosome secretion when changes in their release and composition are observed.

As cargo proteins are thought to be responsible for the recruitment of specific sorting machineries, that in turn seem to dictate the fate of the MVEs where they act, any modification of these cargoes may influence their fate. Indeed, post-translational modification (PTM) of cargo proteins is a major regulator of their intracellular trafficking and sorting to exosomes. Interestingly, different PTMs can compete to determine the fate of the same cargo protein [229]. One important PTM is ubiquitination. As mentioned, ubiquitinated cargoes participate in the recruitment of the ESCRT machinery. This process is particularly important for the sorting of cargo proteins into ILVs fated for lysosomal degradation and can contribute to determine the fate of a given cargo. For instance, ubiquitinated MHC-II is targeted to MVEs fated for lysosomal degradation and sorted to ILVs probably via an ESCRT-dependent mechanism. On the contrary, non-ubiquitinated MHC-II can be secreted on exosomes and in a process that involves its incorporation into CD9-containing membranes, probably in an ESCRT-independent manner [230]. Similarly, ubiquitination seem to negatively regulate the exosomal secretion of the small integral membrane protein of the lysosome/late endosome SIMPLE, given that its secretion is enhanced after mutation of its binding site for ubiquitin ligase [231]. This set of studies strengthens the notion that ESCRT-dependent and -independent mechanisms are respectively associated with exosome release and lysosomal degradation. Nevertheless, ubiquitinated proteins are found in exosomes [197]. Other PTMs that could influence the incorporation of cargo proteins into exosomes are sumoylation (addition of a small ubiquitin-related modifier (SUMO)), as reported for the heterogeneous nuclear ribonucleoprotein A2B1 (hn-RNPA2B1), a key regulator for the recognition of specific miRNAs and their loading into exosomes [232], phosphorylation, as in the case of annexin A2 [218], and oxidation, as in the case of  $\gamma$ -synuclein [233]. A recent paper reported that ubiquitin-like 3 (UBL3)/membrane anchored Ub-fold protein acts as a PTM factor that regulates the sorting of cargoes to exosomes [234]. Moreover the detection of many glycosylated proteins in exosomes suggests that glycosylation may also have a role in exosomal sorting, despite further investigation is needed [229]. Furthermore, palmitoylated cargoes seem to be present on exosomes [235]–[237]. Nevertheless, palmitoylation might be more required for cargo endosomal trafficking than for their sorting into ILVs. Finally, ISGylation, the addition of the small ubiquitin-like protein ISG15, has recently been shown to negatively regulate exosome release [238], although it would act on the sorting mechanism and the fate of the MVE rather than on the cargo protein itself.

In addition to PTM, cargo proteins can undergo proteolytic cleavage before being incorporated into exosomes. In this context, heparanase, the only mammalian enzyme able to cleave heparan sulphate internally, was identified as a stimulator of the syndecan–syntenin–ALIX pathway [239]. The trimming of the heparan sulphate side chains of

syndecans by an endosomal heparanase would favor clustering of syndecans and their proteolytic processing to generate 'SDC-CTF (syndecan C-terminal fragment)', which is sorted to ILVs. Such processing may reinforce the syndecan–syntenin interaction, thereby favoring ILV budding. In melanocytes, the protein PMEL represent another example of coordination between processing and sorting [126], as described above.

In conclusion, any enzyme involved in the modification or processing of exosomal cargo proteins can be considered as a regulator of exosome biogenesis. Nevertheless, the recruitment of a specific sorting mechanism and their relative contribution to exosome biogenesis also depend on the cell type, cellular homeostasis and pathological conditions.

#### **2.4. Balance between recycling and degradation/secretion**

As described above, ILV formation starts in early endosomes, a compartment in which recycling of cargoes also take place. Cargo proteins fated for ILV sorting need to be separated from cargoes fated for recycling and to avoid the recycling pathway. Similarly, cargo proteins that are destined for recycling to the PM will probably not be enriched in exosomes, unless their recycling is impaired, as in the case of the transferrin receptor in reticulocytes [240]. Hence, a balance between cargo recycling and sorting into ILVs can be seen as an additional checkpoint that can influence exosome biogenesis and composition (**Figure 11**).

Among the proteins that regulate this crossroad between recycling and intraluminal sorting, syntenin is an ideal candidate. As described, syntenin interact with syndecans and regulate their sorting into ILVs, but syntenin can also support syndecans' recycling to the PM via a mechanism that requires the production of PI(4,5)P2 and the small GTPase Arf6 [241]. Interestingly, Arf6 and its effector PLD2 also control budding of ILVs and biogenesis of exosomes via the syndecan–syntenin–ALIX pathway [206]. Although it still has to be determined whether recycling of syndecans directly affects syntenin–exosome biogenesis, the fact that the same proteins play a role in both recycling and intraluminal sorting suggests that the two processes have to be tightly balanced. In addition, Arf6 has a main role in the budding of microvesicles from the PM [242, p. 6]. Consequently, one can imagine that by regulating the localization of cargo proteins between endosomes (site of exosome biogenesis) and plasma membrane (site of microvesicle biogenesis) proteins such as Arf6 may control the balance between the generation of this two EV subtypes.

Another pathway that mediates egress of cargoes from endosomes and that can potentially affect exosome biogenesis is the retrograde transport (**Figure 11**). In this context, parkin, a mutated E3 ubiquitin ligase found in Parkinson's disease, seem to regulate such balance between retrograde transport and targeting to ILVs by acting on both retrograde transport (through the retromer complex) and inward budding, and exosome secretion [243]. In addition, sortilin, a receptor involved in anterograde transport from the Golgi body and a known cargo protein of retrograde transport, has also been shown to be secreted on exosomes and to modulate exosome biogenesis [244], although the trafficking mechanisms underlying such regulation need further investigation. Finally, deficiency of the retromer

complex have been associated to an increased secretion of APP C-terminal fragment in exosomes [245].

A similar balance between retrograde pathway and degradative pathway also exist. For instance, in case of retromer deficiency, cargoes such as CI-M6PR or MIG-14/Wntless are sorted for lysosomal degradation.

Considering that cargoes fated for recycling/retrograde pathways and degradative/exosome pathways coexist in early endosomes, it is clear that mechanisms that segregate such cargoes and maintain specific subdomain for retrieval or ILV sorting must exist. I have already discussed a possible role for actin in avoiding later diffusion of cargoes and their entrance in a degradative microdomain, and actin role in the maintenance of the recycling tubular domain. Another protein that could contribute to the separation of the “degradative domain” from the “recycling domain” is the J-domain protein RME-8. RME-8 is an interactor of SNX1 and therefore it participates to cargo recycling to the Golgi. RME-8/SNX1 positive microdomains appear to be separated from HRS/ESCRT positive microdomain in *C. elegans* endosomes. While knockdown of HRS had no effect on the RME-8/SNX1 microdomain, mutation of RME-8 or SNX1 increased the extension of the HRS microdomain, illustrating how the recycling machineries can control the degradative machine [246].

### **2.5. Balance between lysosomal degradation and secretion of MVEs**

The main fate of an MVE is to fuse with lysosomes and degrade their content. Therefore, MVEs must avoid lysosomal degradation in order to release their ILVs as exosomes. This step represents another critical checkpoint for exosome secretion that may be particularly relevant in pathological conditions (**Figure 11**). The existence of a balance between lysosomal degradation and exosome secretion is supported by the observation that the inhibition or the blocking of lysosomal function induces exosome secretion. For instance, treatment with bafilomycinA1 or concanamycin, two lysosome inhibitor, increases EV secretion [238], [247][292] or exosomal secretion of specific cargo proteins such as  $\alpha$ -synuclein [249]. On the same line, inhibition of V-ATPase in *Caenorhabditis elegans* has been shown to trigger apical secretion of the Hedgehog protein [250]. In a recent study lysosomal activity was impaired by the use of chloroquine resulting in increased exosomal secretion [251]. Finally, depletion of Sirtuin 1, a protein down regulated in breast cancer, that seem to impair V-ATPase expression and impair lysosomal function, results in the secretion of exosomes with unique cargoes that promote cancer progression [252]. As mentioned, lysosomal function is affected in several pathologies such as lysosomal storage diseases or neurodegenerative diseases. In the case of NPC disease, one study showed that NPC1 mutation or the use of U18666A inhibitor enhances secretion of cholesterol in exosomes [253]. Similarly, impairment of lysosome function in neurodegenerative diseases and consequent intracellular accumulation of toxic proteins seem to provoke their extracellular secretion [254]. Accumulations of APP fragments A $\beta$  and tau are typical features of Alzheimer’s disease. Both proteins have been found in EVs released from cell culture models of Alzheimer’s disease, in EVs isolated from cerebrospinal fluid, brain extracellular space and

blood samples from patients with Alzheimer's disease [255], [256]. Despite so far there is still no direct evidence of a causal connection between intracellular accumulation and the release of these pathological proteins in EVs, secretion of these toxic products would certainly compensate for their impaired degradation inside the cells, thereby contributing to the maintenance of cellular homeostasis. This set of studies also strengthens the first studies on exosomes [257] that considered exosomes as a way of eliminating obsolete or potentially toxic compounds that had accumulated in cells.

Nevertheless, the mechanisms that prevent lysosomal degradation in favor of exosome secretion are still poorly understood. As suggested above, ESCRT-independent and -dependent sorting seems to drive MVEs to either secretion or lysosomal degradation respectively. A recently identified mechanism involves the post-translational modification of endosomal proteins. In particular, ISGylation of TSG101 [238] inhibits exosome secretion whereas mutations that impair ISGylation enhance exosome secretion. Such regulation is counterbalanced by the fusion of MVEs with lysosomes, establishing the first mechanism that could decide the fate of an MVE, hence fusion with either a lysosome or the PM.

It also supports the notion that the first subunits of the ESCRT mechanism could be used by secretory and degrading MVEs; it would therefore be interesting to know whether ISGylation of TSG101 determines recruitment of either the conventional ESCRT machinery for degradation or ALIX for secretion. In addition, Tetraspanin 6 (Tspan6) has recently been involved in the regulation of the balance between lysosomal degradation and exosomal secretion. Overexpression of Tspan6 slows down lysosomal degradation of APP-CTF and enhances its secretion on exosomes probably by recruiting syntaxin [258, p. 6].

Despite these recent reports, it is still not clear how and if the mechanisms of sorting would control the fate and function of MVEs. One hypothesis implies the presence of distinct subsets of MVEs fated for degradation or for secretion where ESCRT-dependent and ESCRT-independent mechanisms would be exclusively active. Alternatively, ESCRT-dependent and -independent sorting pathways could act together on the same MVE, and the abundance of a given cargo protein or its PTM will favor the recruitment of one sorting mechanism over another and dictate the destiny of the MVE. Finally, as observed in specialized cells such as melanocytes, ESCRT-independent and -dependent sorting could act sequentially, with the former acting first whereas cargo proteins destined for the latter are sequestered into a clathrin coat at the limiting membrane. In this case it is likely that only early MVEs would be targeted for secretion. Considering that evidences in favor of each hypothesis exist, it is likely that the predominant model would depend on the cell type and condition. For instance, dendritic cells use different sorting mechanisms according to their maturation stage and reticulocytes use different mechanisms for exosome secretion while maturing into erythrocytes. Finally, the only feature that has allowed discrimination of subpopulations of MVEs with apparent distinct destinies is their cholesterol content [182]. This observation is very intriguing considering the role of cholesterol in the regulation of late endosome motility.

As mentioned above, MVEs can fuse with autophagosomes to form an amphisome. This organelle usually fuses with lysosomes for degradation of its content, in a process called macroautophagy. Despite a lack of data on the potential link between the macroautophagy signalling pathway and exosomes, there may be a similar balance between exosome secretion and autophagy which would depend on external stimuli such as starvation (**Figure 11**). In support of this hypothesis, it has been shown that the prion protein (PrP) can promote exosome secretion by inhibiting autophagosome formation through interaction with caveolin 1, a suppressor of autophagosome formation [259]. Moreover, inhibition of the kinase PIKfyve, which also participates in the regulation of autophagy, increases secretion of a subpopulation of 'autophagic' EVs enriched with autophagic proteins, but not with 'classic exosomal markers', again suggesting that impairment of autophagy can be balanced by secretion [260]. Interestingly different autophagy proteins have recently been shown to regulate exosome secretion, such as the ATG12–ATG3 complex which controls both autophagy and exosome secretion via interaction with ALIX [261], or ATG5 which induces sequestration of a subunit of V1V0-ATPase in ILV, inhibiting in this way ATPase activity, and promotes MVE acidification and exosome secretion [262, p. 5]. In addition, the tetraspanin CD63 has recently been proposed to coordinate both autophagic and endosomal processes and to regulate exosomal secretion of EBV-encoded LMP1 [263]. Finally, it is noteworthy that lysosomes and autolysosomes can also fuse with the PM and secrete their content, including any remaining ILVs, which would be impossible to distinguish from MVE-derived exosomes after secretion. Even though these pathways could use different mechanisms, they would all contribute to the maintenance of cell homeostasis and probably share common features.

## **2.6. Transport of MVEs to the plasma membrane and exosome release**

Regardless of their fate, MVE transport is required in order to promote fusion with lysosomes or the PM. As in the case of transport vesicles, intracellular transport of organelles requires association with the cytoskeleton, the action of molecular motors and small GTPases [264], while their fusion involves SNARE proteins [119] (**Figure 11**).

Several Rab proteins have been involved in exosome secretion. Depletion of early endosomal Rabs, such as Rab11 and Rab35, have been shown to decrease exosome secretion or secretion of specific exosomal cargoes both in cell lines and in vivo models [265]–[268]. Other studies confirmed the involvement of Rab35 in exosome secretion from primary oligodendrocytes [269] or oligodendroglial cell lines, where Rab35 seems to have a role in the docking and tethering of MVEs to the PM [270]. An shRNA-based screen in HeLa cells expressing MHC-II molecules showed that silencing of Rab2B, Rab5A, Rab9A, Rab27A and Rab27B decreased CD63, CD81 and MHC-II secretion in exosomes [226]. In particular, this study showed the involvement of RAB27A/B in the docking of MVBs to the PM. This role has been confirmed in many cell lines and Rab27A silencing is commonly used as a way of modulating exosome secretion. Finally, Rab7 has been involved in the secretion of syntenin–ALIX exosomes by MCF-7 cells [205], whereas its depletion does not affect exosome

secretion in HeLa cells [226]. The fact that both early and late endosomal Rab proteins have a role in exosome secretion suggests that, according to the cell type, different subpopulations of MVEs originating either from early or recycling endosomes or from late endosomes are used to release ILVs as exosomes. Alternatively, Rab proteins, except for the ones involved in the docking process, may regulate exosome secretion indirectly by maintaining endosomal homeostasis.

The importance of the cytoskeleton in exosomes secretion is supported by the observation that exosome released can take place in specific location of the PM, as in the case of immunological synapses in antigen-presenting cells [230], [271] or invadopodia [202] in cancer cells. In this case, the microtubule network would be of primary importance for the transport of MVEs to their site of secretion. As aforementioned, Rab7 and its effector RILP modulate the transport of late endosomes and in consequence their fusion with lysosomes, through a process that also depends on the levels of cholesterol in the limiting membrane of MVEs [272], [273]. This suggests that the composition of the limiting membranes of MVEs may modulate their destiny by acting on the motility of MVEs. If we consider the sorting of cargoes into ILVs as a way to modulate the composition of the delimiting membrane of MVEs, it is clear that ILV biogenesis may in turn have a consequence on the fate of MVEs. In addition to microtubule cytoskeleton, actin cytoskeleton also contributes to exosome release, because it is required for the docking and fusion of MVEs with the PM. The actin regulatory protein cortactin has been shown to promote exosome secretion by regulating both trafficking and docking of MVEs to the PM together with the Rab27A and coronin1b [274].

Despite the mechanisms involved in the docking and fusion of MVEs with the PM are not completely understood, some Rab GTPases, SNARE proteins and synaptogamin family members participating to this process have been identified. As already mentioned, Rab27A and Rab27B (together with their respective effectors, synaptotagmin-like protein 4 and exophilin 5) mediate the docking of MVEs to the PM in order to promote their fusion [226] in concert with the actin cytoskeleton [274]. The SNARE protein VAMP7 has been involved in exosome secretion in the leukemia cell line K562 and expression of a form of VAMP7, which inhibits SNARE complex formation, decreases exosome secretion [275]. Another SNARE protein, YKT6, is required for exosome release in HEK239 cells [201] and A549 lung cancer cells [276]. In *C. elegans*, the Ras-related GTPase homologue Ral-1 is involved in MVE biogenesis and their fusion with the PM together with the SNARE protein, syntaxin 5 [277]. Recently, phosphorylated SNAP23 has been shown to enable exosome release [224], [278]. In this context, the release of Ca<sup>2+</sup> into the cytoplasm seems to be not always required for the activation of the SNARE complex involved in MVE–PM fusion, revealing a cell type-specific contribution of this known regulator of exosome secretion [224], [279]–[281].

In conclusion, exosome secretion will determine exosome functions because it will direct exosome release towards either the correct cellular partner, as in the case of a synapse [230] or the correct body fluids or biological barrier for reaching its final destination.

## **2.7. EV secretion: constitutive or regulated process**

The presence of exosomes in cell culture media from cells under basal conditions suggests that exosome secretion can be a constitutive process. Nevertheless, external or internal stimuli received by the producing cells can modulate basal exosome biogenesis and/or secretion.

As mentioned earlier, several studies have shown that release of Ca<sup>2+</sup> in the cytoplasm can regulate exosome secretion in specific cell types. Moreover, pathological conditions such as inflammation or cancers can also modulate exosome secretion or their composition, likely through external stimuli that induce specific intracellular signalling. In the context of inflammation, stimulation of intestinal cells with IFN- $\gamma$  up-regulates the expression of MHC-II and its secretion on exosomes [227]. In addition exosomal release of MHC-II by dendritic cells is stimulated by their interaction with CD4<sup>+</sup> T-cells [230], showing the importance of intercellular communication in the modulation of exosome release. In the context of cancer, the release of exosomes, visualized using a CD63-pHluorin tool, is stimulated by histamine, a ligand of GPCRs, via a signaling pathway that involves protein kinase C [224]. Furthermore, mutations in KRAS, which frequently occurs in cancer and impair the MEK–ERK pathway, modulate release of Ago2 and specific miRNAs in exosomes [193], whereas HER2 amplification in breast cancer induces the secretion of EVs enriched in proteins that can promote malignant transformation [282]. Recently the cytoplasmic tyrosine kinase SRC has been shown to stimulate secretion of syndecan–syntenin-positive exosomes with pro-migratory capacity [283], through the phosphorylation of specific sites of syndecans and syntenin. Another recent study identified the interaction of the membrane associated tyrosine kinase c-SRC with ALIX in endosomal membranes showing that this interaction would promote exosome secretion in cancer cells [284]. Finally, chemotherapy can be seen as an external stimulus that modulates exosome secretion by cancer cells. These exosomes, once secreted, mediate resistance to chemotherapy, mostly by two mechanisms. First, exosomes can present a way for cells to remove drugs from their cytosol, as shown in the case of cisplatin-resistant ovarian carcinoma cells [285]. Interestingly, these cells seem to have an abnormal lysosomal compartment compared with cisplatin-sensitive cells, suggesting that exosomal secretion of cisplatin could compensate for lysosomal impairment. Second, exosomes secreted by resistant cells can transfer active molecules such as miRNAs to sensitive cells, so inducing resistance [286].

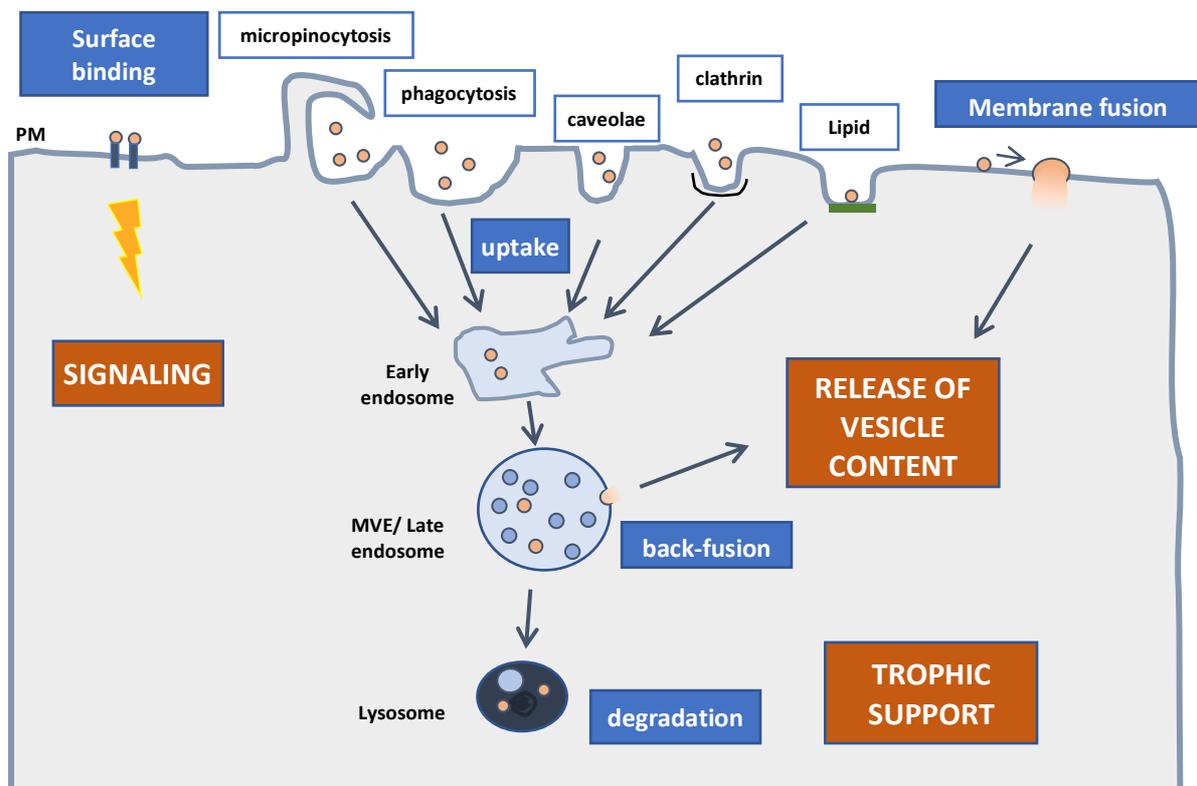
## **2.8. Clinical applications of EVs**

Once released in the extracellular environment, EVs can reach body fluids, such as blood, and be transported to their target cells far from their site of production. Indeed, EVs have been found in all kind of body fluids, including blood, lymphatic circulation, cerebrospinal fluid, saliva and urine. EVs released in body fluids of healthy patient and patients with different diseases display different protein and RNA content that can be measured and used as potential biomarkers of specific diseases [287]. Isolation of EVs from biological fluids and

their use as biomarkers is nowadays of great interest, given that the use of liquid biopsies would represent a non-invasive alternative to surgical procedures to obtain tissue biopsies. Finally, the fact that EVs can be transported to recipient cells through body fluids is of great interest for therapeutic application of EVs, with the idea that EVs could deliver their cargoes to specific cell types to promote or restore their functions. Indeed, EVs naturally released by specific cell types, such as mesenchymal stem cells or immune cells, display a therapeutic potential and could be used in case of pathological conditions. Alternatively, EVs can be loaded with exogenous cargoes, such as therapeutic miRNAs or small drug molecules. In any case, the main challenge is represented by the specific targeting of EVs to their target tissues, given that the biodistribution of EVs after their systemic administration can drastically affect their effect on recipient cells. In this context, the development of new in vivo models and of new techniques to track EV distribution and targeting to recipient cells is definitely needed and is emerging with the development of new model organisms such as zebrafish [288].

### 2.9. EV uptake and cargo delivery to recipient cells

Once at their destination, EVs need to be recognized and captured by target cells in order to induce phenotypic changes (Figure 13).



**Figure 13. Fate of EVs in recipient cells.**

EV can bind specific receptors at the surface of the recipient cells and induce a signaling cascade, without delivering their content. EVs can also be internalized through several mechanisms and transported to late endosomes. Fusion of late endosomes with lysosomes

would allow the degradation of EVs. EV components may be used by the cells as trophic support. Alternatively, back-fusion of EVs with the delimiting membrane of endosomes or direct fusion of EVs with the plasma membrane of recipient cells allows the release of EV intraluminal content into the cytoplasm. (adapted from van Niel et al, 2018).

Of note, EVs can exert their function not only on cells that are far from their producing cells (e.g. other organs), but also on cells that are in close proximity. In the latter case, EVs can remain closely associated to the plasma membrane of their producing cells through a mechanism involving the transmembrane protein tetherin [247].

Independently of their site of action, EVs can exert an effect on recipient cells without delivering their content but remaining at the plasma membrane and inducing a signaling cascade. This is the case of the activation of immune T cells by exosomes bearing MHC-II [289]. Alternatively, EVs can be internalized and deliver their cargoes. The mechanisms that mediate the recognition of a given EV subtype by specific cell types are still not clear. Different studies illustrated the specificity of EV uptake by showing that specific subpopulation of EVs are taken up by some cell types and not by others. For instance, small EVs released by oligodendrocytes are preferentially internalized by microglia but not by neurons [290]. Similarly, EVs released by cortical neurons are only internalized by neurons, while EV release by neuroblastoma are internalized by both neurons and glial cells [291]. On the contrary, HeLa cells can internalize EVs derived from different cell types, suggesting that internalization may also not require specific recognition. At the moment, the mechanisms of EV uptake are also not completely understood. Internalization may occur through nonspecific mechanisms such as pinocytosis or being mediated by receptors that will recognize specific EV cargoes [292]. In addition, it is not clear if internalization of EVs could occur everywhere on the cell surface or in specific region of the plasma membrane. One report suggested that internalization would preferentially occur in proximity of cell protrusions such as filopodia [293]. So far, different mechanisms for EV endocytosis have been proposed, including clathrin-mediated, caveolin-mediated, lipid-raft mediated endocytosis, phagocytosis and micropinocytosis. Regarding the receptors that would mediate endocytosis and the recognized molecules on EVs, several candidates have been proposed. These include integrins and tetraspanins at the surface of EVs, heparan sulfate proteoglycans or scavenger receptors at the surface of recipient cells and the protein TIM4, which recognize phosphatidylserine on the surface of EVs [294], [207], [295], [296], [297, p. 4]. Alternatively to the uptake, EVs can deliver their content to recipient cells through direct fusion with the plasma membrane [298]. A process of fusion would also be required for the delivery of EV content in the cytoplasm upon internalization, in this case EVs internalized by the endocytic pathway would deliver their content to the cytoplasm by back-fusion with the delimiting membrane of endosomes. A recent report visualized this process by fluorescently labeling exosomes with the dye rhodamine C18, a dye used to study virus-induced membrane fusion [299]. The fluorescence of this dye is initially quenched in exosomes due to its high concentration, while it is dequenched when the dye diffuses in membrane as in

the case of a membrane fusion event. This report also suggest a role in this process for LBPA, that was already reported as a possibly mediator of the fusion of late endosome internal membrane with the delimiting membrane together with ALIX [300], [301]. Nevertheless, the main fate of endocytosed EVs seem to be their degradation in lysosomes. In this case recipient cells may use EV components as trophic support. Overall, the development of new techniques to study EV cargo delivery into recipient cells will definitely shed new light on these mechanisms.

In conclusion, independently of the pathways used for recognition and cargo delivery, EVs exert many different functions in both physiological and pathological conditions that have been extensively reviewed elsewhere [302], [303].

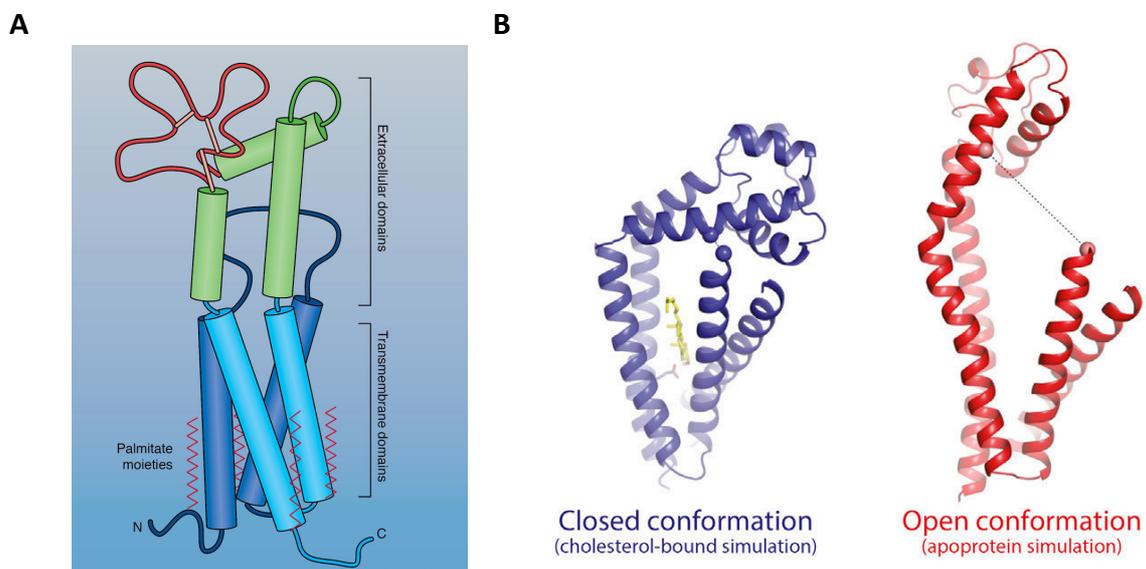
EVs are a heterogenous group of cell-derived membrane vesicles.  
Biogenesis of exosomes is regulated by various checkpoints throughout the endosomal pathway.  
The intracellular pathways involved in EV biogenesis determine their function because they regulate EV composition, specific release and fate.

### 3. CD63

Given that the main aim of my thesis was to further elucidate the role of the tetraspanin CD63 in exosome biogenesis and in endosomal homeostasis, in this section I will illustrate the properties and the roles of CD63.

#### 3.1. Tetraspanins and tetraspanin enriched macrodomains

Tetraspanins are a large family of transmembrane proteins widely expressed in metazoans, but also in fungi and plants. In particular, 33 different tetraspanins are found in humans. Tetraspanins are characterized by the presence of four transmembrane domains (TM), a small extracellular loop (EC1), a large extracellular loop (EC2) and three short intracellular regions (**Figure 14**) [304].



**Figure 14. Structure of tetraspanins.**

**A.** Tetraspanins present four transmembrane domains and two extracellular domains. Structurally conserved subdomains in the large extracellular loop (EC2) are indicated in green, while structurally variable subdomain stabilized by disulfide bonds are indicated in red. (from Charrin et al, 2014).

**B.** Model of the crystal structure of CD81. Cholesterol (in yellow) is allocated in an intramembrane cavity within the transmembrane regions, while the EC2 assume a closed conformation (in blue). In absence of cholesterol the EC2 assume an open conformation (in red). (from Zimmerman et al, 2016).

Despite the extracellular loops show great divergency among different tetraspanins, the EC2 region contains four or six conserved cysteine residues. These residues form intramolecular disulfide bonds crucial for the correct folding of EC2. Very less is known about the EC1, except that it contains glycosylation sites in some tetraspanins, such as CD9. On the contrary, the structure and functions of EC2 are much more characterized. Structural analysis of the EC2 of CD81 and molecular modelling [305], [306] illustrated the presence of

three  $\alpha$ -helices that are stabilized by the disulfide bonds. These regions are probably present in all tetraspanins and may mediate tetraspanin homodimerization. Other two  $\alpha$ -helices and flanking sequences constitute the variable region of the EC2, that is not conserved among tetraspanins and mediates specific protein-protein interactions. In addition, the modelling suggested that the EC1 fits into a loop of the EC2 and that the transmembrane domains form a coil-coiled structure stabilized by hydrogen bonds [306]. Interestingly, the crystal structure of CD81 have been recently determined revealing a cone-like architecture of this tetraspanin and the presence of a large intramembrane cavity within the transmembrane region in which cholesterol can be allocated [222]. Molecular dynamics simulations in the same study also suggest that the EC2 can assume an open and a close conformation, respectively in the absence and in the presence of cholesterol in the cavity (**Figure 14**). Finally, tetraspanins are glycosylated to variable extents and palmitoylated on cysteine residues of their intracellular domains.

A key role of tetraspanins consist in the organization of the so-called “tetraspanin web” or “tetraspanin enriched microdomains” (TEM) in which different tetraspanins interact with each other and with a network of different partner proteins. Despite TEMs were mostly found at the plasma membrane, similar microdomains may be present in endosomes, where tetraspanins such as CD63 are highly enriched. Within this web, we can distinguish primary complexes in which tetraspanin directly and specifically interact with other proteins, and secondary interactions in which tetraspanins associates with each other or with non-tetraspanin proteins indirectly via other proteins. These secondary interactions are also stabilized by tetraspanin palmitoylation, hence illustrating the fundamental role of these post-translational modification in the maintenance of these microdomains [307]–[309]. Finally the organization of TEMs is regulated by lipids such as cholesterol and gangliosides and the palmitate moieties of tetraspanins [310], [311]. For this reason, TEM are resistant to solubilization in non-ionic detergents and the use of mild detergents (e.g. Brij99 or CHAPS) has allowed the identification of several multiprotein complexes in TEM. The main tetraspanin protein partners are integrins, adhesion molecules and metalloproteases. These classes of transmembrane proteins mostly interact directly with the EC2 of tetraspanins. On the contrary cytosolic proteins, mostly signaling proteins, can interact directly with tetraspanin intracellular domains. Moreover, the intracellular domain of CD9 and CD81 can interact with the actin linking proteins ezrin-radixin-moesin, directly or indirectly, hence connecting TEM to the actin cytoskeleton [312]. In conclusion, because of their interaction with different classes of proteins, tetraspanins have been involved in many cellular processes including the regulation of intracellular trafficking and signaling pathways, cell adhesion and migration, cell-cell interaction and cell fusion.

### **3.2. CD63**

CD63, also known as LAMP-3 or LIMP-1 or Tspan-30 or melanoma-associated antigen ME491, was the first characterized tetraspanin, originally discovered as a protein on the cell surface of platelets (and called platelet glycoprotein 40) and as a melanoma antigen [313].

CD63 is ubiquitously expressed and at steady state is mostly localized within the endosomal system, especially in late endosomes/MVEs, lysosomes and LROs. In addition, CD63 is also found at the cell surface. CD63 interact with several proteins, including other tetraspanins, integrins, cell surface receptors, signaling proteins and ion transporters.

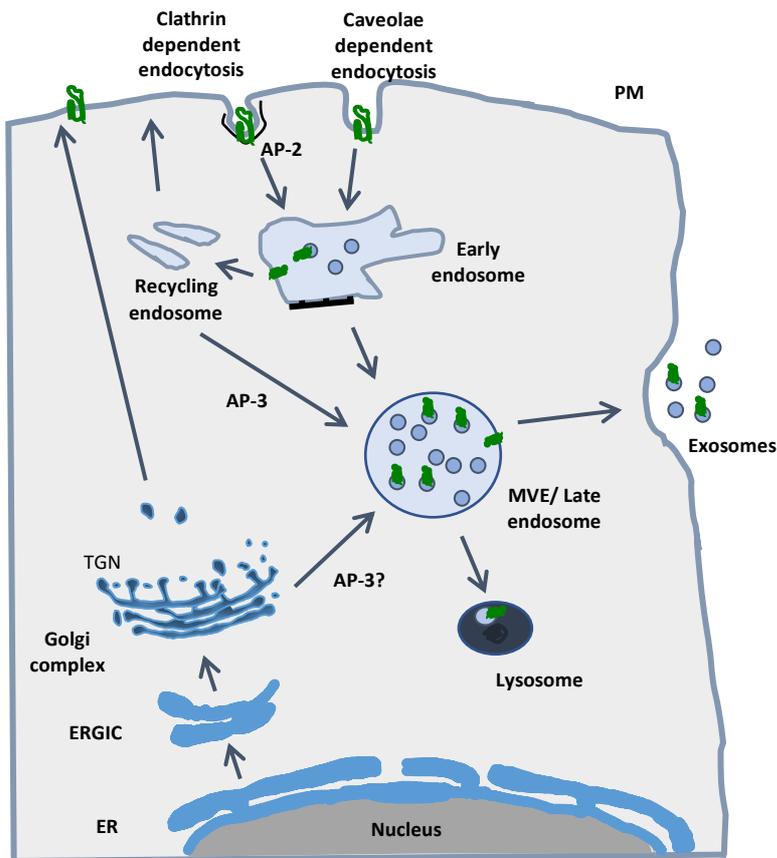
Despite the crystal structure of CD63 has never been reported, CD63 as all other tetraspanins present four transmembrane domains, a small and a large extracellular loop and three cytosolic domains. CD63 is heavily glycosylated, therefore when non-reducing conditions are used for Western Blot, CD63 appear as a smear between 25KDa and 70 KDa.

### **3.3. Intracellular trafficking of CD63**

The carboxy-terminal cytosolic region of CD63 contain a tyrosine-based motif (YXXØ) required for the lysosomal targeting of CD63 [48]. This motif is recognized by two adaptor proteins: AP-2 and AP-3. Despite the intracellular trafficking of CD63 has not been yet completely elucidated [314], it is believed that after exiting the TGN, CD63 is transported to the PM and reinternalized through AP-2 mediated clathrin dependent endocytosis (**Figure 15**). Nevertheless, depletion of AP-2 blocks only partially the sorting of CD63 to late endosomes, suggesting that a direct pathway TGN-endosomes can exist. This pathway would be independent of the classical AP-1 pathway, given that silencing of AP-1 do not alter the localization of CD63 [315]. As previously described, AP-3 is localized at early endosomes, where it contributes to the transport of proteins to lysosomes and LROs [142], [316]. In addition, depletion of AP-3 induced a faster recycling of CD63 to the PM [316]. AP-3 could then contribute to the trafficking of CD63 from early endosomes to late endosomes/lysosomes and LROs (**Figure 15**). In addition, AP-3 may be involved in the direct transport of CD63 from the TGN to late endosomes/lysosomes, nevertheless this pathways has not been well characterized yet [317]. Alternatively to clathrin mediated endocytosis, CD63 may be internalized via caveolae (**Figure 15**), given it has been observed in caveolae at the plasma membrane [314].

Contrarily to LAMP1, CD63 is mainly localized on ILVs of MVEs and not on the delimiting membrane. Hence, ILV sorting of CD63 can also be seen as a transport pathway of CD63 from early endosomes to late endosomes during endosomal maturation.

Finally, a small portion of CD63 localize in the plasma membrane, this portion may represent CD63 on the way to endosomes, but it has been shown to have specific roles, such as interaction with integrins and regulation of their internalization.



**Figure 15. Intracellular trafficking of CD63.**

CD63 is transported from the TGN to either the plasma membrane or to endosomes via a direct pathway. Internalization of CD63 from the cell surface occurs through AP-2/clathrin mediated endocytosis. After endocytosis CD63 is transported to late endosomes/lysosomes via sorting into ILVs or via recycling endosomes, in a process mediated by AP-3. Finally, fusion of MVEs with the plasma membrane allows secretion of CD63 on exosomes.

### 3.4. CD63 interacting proteins

Different interactors of CD63 have been identified and they contribute to CD63 functions in different cell types. First, CD63 directly interacts with syntenin [223]. This interaction requires the carboxy terminal domain of CD63 and the PDZ domain of syntenin. Although CD63 can interact with both PDZ1 and PDZ2 of syntenin, the interaction with PDZ1 is much stronger. In addition, a region in the carboxy terminal region of syntenin, located after the PDZ2, is required for the stabilization of the interaction with CD63. Despite CD63 and syntenin also colocalize in endosomes, the complex CD63-syntenin seems to be abundant at the plasma membrane. Interestingly, the carboxy terminal domain of CD63 contains the tyrosine-based motif recognized by AP-2 for its internalization. Moreover, overexpression of syntenin reduced the internalization rate of CD63, suggesting that syntenin may compete with AP-2 for the binding of CD63. Hence, syntenin may indirectly regulate the trafficking of CD63.

Considering that syntenin also interact with signaling proteins, the interaction CD63-syntenin may mediate the indirect interaction of tetraspanin enriched microdomains and signaling pathways. CD63-syntenin interaction may also have a role in exosome biogenesis. Indeed, CD63 have been found to be a cargo of the syndecan-syntenin-alix pathway [205]. This study and our observations (in the Result section) suggest that CD63 is not required for the sorting of syntenin into exosomes, while syntenin seem to be required for the sorting of CD63 into exosomes. Finally, the interaction CD63-syntenin is relevant in pathological conditions, for instance in the regulation of the post-endocytic trafficking of oncogenic human papillomaviruses [318].

The L6-antigen, that is overexpressed in many epithelial cancers, has also been shown to be recruited in CD63 positive TEM at the plasma membrane [319]. Interestingly, depletion of L6-antigen increased the surface expression of CD63. Despite this regulation is not well characterized, it can have implications not only on the localization of CD63 but also in the organization of TEM and in cancer cell migration. In the context of cancer cell migration, interaction of CD63 with integrins, with inhibitor of metalloproteases and metalloproteases are also relevant. More specifically CD63 interacts with Integrin  $\beta 1$  and with the tissue inhibitor of metalloproteases-1 (TIMP-1) [320]. The formation of this tripartite complex has been shown to promote survival of melanoma cells [321] and to promote the formation of a pre-metastatic niche in the liver for the dissemination of pancreatic cancer [322]. Finally, CD63 interact with the membrane-type 1 matrix metalloproteinase (MT1-MMP) and seem to regulate its lysosomal targeting and degradation [323].

In immune cells CD63 interact with MHC-II, hence participating in immune cell functions. First, in antigen presenting cells MHC-II is exposed to the cell surface prior to the formation of the immunological synapse through its integration in specific membrane domains such as lipid raft or CD63 containing TEM [324]. In addition to this colocalization in the PM, CD63 is enriched in MHC-II containing MVEs, called MIIC [325] and may participate in the internalization of MHC-II in immature dendritic cells [326]. Moreover, secreted exosomes bearing MHC-II and tetraspanins are able to induce T-cell activation [289], [327], [328]. Interestingly CD63 expression seem to modulate the release of MHC-II containing exosomes and the subsequent activation of T-cells [328].

Finally, CD63 interact with a type II phosphatidylinositol 4-kinase (PI4KII), likely PI4KII $\alpha$ , both in TEM at the plasma membrane and intracellularly [329], [330]. The role of this interaction is not known, but CD63 may contribute to the recruitment of PI4K in specific cellular locations and influence PI dependent signaling and PI metabolism.

### **3.5. Role of CD63 in intracellular trafficking**

In addition to the aforementioned interactions, CD63 participates to cellular functions by regulating the intracellular trafficking of other proteins. I have already described the role of CD63 in the sorting of the melanosomal protein PMEL into ILVs in melanocytes [139]. In this case CD63 interact with the C-terminal domain of PMEL suggesting that CD63 may

contribute to the maintenance of PMEL in specific subdomains of the endosomal delimiting membrane to allow PMEL correct processing and sorting.

In macrophages CD63 regulates the trafficking of the  $\text{Ca}^{2+}$  sensor Synaptotagmin VII (Syt VII) to lysosomes and phagosomes [331]. The YXXØ motif of CD63 is needed for Syt VII lysosomal sorting, illustrating how proteins that do not contain specific sorting motifs associate with other proteins to reach their correct localization. Moreover, this interaction is dependent on Syt VII palmitoylation, illustrating the importance of palmitoylation in the formation of a tetraspanin microdomain. On the contrary, palmitoylation of CD63 was not required for Syt VII interaction and trafficking. Another post-translational modification of CD63, N-glycosylation, is required for the interaction of CD63 with the chemokine receptor CXCR4. This interaction takes place in the Golgi apparatus and is required for the trafficking of CXCR4 toward endosomes/lysosomes instead of the plasma membrane [332]. Moreover, CD63 has been involved in the internalization of the ion pump H, K-ATPase  $\beta$ -subunit in gastric parietal cells and its transport to a tubulo-vesicular storage compartment, through CD63 interaction with AP2 or AP3 [333]. CD63 also exert important function in the kidney, by regulating the transport of ion channels. In the case of the renal outer medullary potassium channel, ROMK1, this regulation is indirect with CD63 associating with receptor-linked tyrosine phosphatase  $\alpha$  (RPTP $\alpha$ ) and activating c-SRC signaling that in turn regulate ROMK1 endocytosis and activation [334]. A recent study reported the interaction of CD63 with the human organic cation transporter 2 (hOCT2) and the participation of CD63 in the basolateral distribution of hOCT2 in polarized cells and in the renal proximal tubule [335]. Overall, the function of CD63 in the kidney are a relevant example of the in vivo functions of CD63, given that CD63 knock-out mice show kidney pathology and altered water balance [336].

### **3.6. Role of CD63 in LROs**

CD63 is found in various lysosome-related organelles including melanosomes in melanocytes, Weibel-Palade bodies in endothelial cells, primary granules in neutrophils, cytolytic granules in T cells, dense and alpha granules in platelets, basophilic secretory granules in mast cells. Despite the presence of CD63 is a common feature on all LROs, the role of CD63 in these organelles is not well identified.

Weibel-Palade bodies are LROs found in vascular endothelial cells that store the von Willebrand factor, they originate from the Golgi apparatus but they also receive proteins such as CD63 and P-selectin from late endosomes [337]. In particular the trafficking of CD63 to WPBs is mediated by AP-3 [338]. The presence of CD63 on the delimiting membrane of WPBs leads to its deposition on the plasma membrane of endothelial cells upon secretion of these LROs induces. This process contributes to the activation of endothelial cells that leads to an inflammatory response and to the recruitment of leukocytes by P-selectin. CD63 deficient cells or mice fail to recruit leukocytes suggesting that CD63 act as a co-factor for P-selectin [339]. Interestingly, a recent study showed that WPBs contain intraluminal vesicles enriched in CD63 that are released in the extracellular environment upon fusion of WPBs

with the plasma membrane [340], pointing out that LROs are secretory organelles and a possible source of extracellular vesicles.

Neutrophils store the serine protease neutrophil elastase in LROs, called primary granules, that are positive for CD63. While the trafficking of CD63 to these granules is dependent on AP-3, the transport of the precursor of neutrophil elastase is regulated by its association with CD63 [341].

Finally, CD63 is very abundant in melanosomes of melanocytes. As described above, previous work from my host lab showed the importance of CD63 in the early biogenesis of melanosomes. Nevertheless, the role of CD63 in mature melanosomes is not known. Mature melanosomes also contain ILVs, that could be secreted together with the melanocore but their role has not been determined yet. At the same time the secretion of melanosomes could induce the deposition of CD63 on the plasma membrane. Hence, further studies of melanosome transfer from melanocytes to keratinocytes in more physiological systems (e.g. coculture, 3D reconstructed skin) could help to elucidate the role of CD63.

### **3.7. Tetraspanins and CD63 in EVs**

As described above, tetraspanins and CD63 are main components of extracellular vesicles. Apart from their role in EV biogenesis, tetraspanins contribute to the function of EVs in cell-cell communication. So far, only few studies have addressed the role of tetraspanins in mediating EV recognition and uptake by target cells. For instance, exosomes bearing a Tspan8-CD49d complex preferentially bind to endothelial cells [211]. Another study illustrates how different tetraspanin webs can influence the targeting of exosomes to different cell type and tissues in vivo [294]. Importantly, tetraspanin enriched microdomains contain integrins or adhesion molecules that also participate to exosome targeting to specific sites. Regarding exosome uptake or fusion with the plasma membrane, the role of tetraspanin is not known. Of note, TEM have been implicated in processes of membrane fusion [342], hence it is tempting to hypothesize that tetraspanins could mediate membrane fusion with target cells.

Given their enrichment in EVs, tetraspanins are largely used as EV markers and reporters. For instance, tetraspanins can be used for EV isolation using immunoprecipitation, for EV detection in flow cytometry using beads conjugated to anti-tetraspanin antibodies, for qualitative analysis of EV composition by Western Blot or immuno-electron microscopy. Expression of fluorescently tagged tetraspanins (e.g. CD63-GFP) is also broadly used to study the release and the uptake of EVs both in vitro and in vivo. Recent works from my host laboratory and the Pegtel's laboratory have developed a tool for the visualization and quantification of exosome release based on a PH-sensitive GFP, the CD63-pHluorin, that can be used both in vitro and in vivo [207], [224]. Alternatively, tetraspanins can be conjugated to the reporter enzyme Luciferase or Nanoluciferase, that is also a suitable tool for in vivo studies [343] or for in vitro quantification of exosome release [248], [344]. Nevertheless, it

should be considered that such strategies may restrict the analysis to only some of the existing EV subpopulations.

### **3.8. CD63 in pathophysiological processes**

Considering its physiological roles, CD63 can also contribute to the development of different pathologies. CD63 knockout model mice do not present gross morphological abnormalities, they are viable and fertile. Despite the role of CD63 in the immune system, no major defects are observed. Nevertheless, these mice display kidney pathology and have an altered water balance, in addition to defective pigmentation [336]. Overall, it is likely that, in vivo, at least some of the CD63 functions are compensated by the expression of other tetraspanins.

As mentioned, CD63 was first discovered as an antigen of early stage melanoma cells [313]. CD63 expression is reduced in more invasive stages of melanoma, suggesting a negative correlation between CD63 expression and melanoma invasiveness. Depletion of CD63 in melanoma cells resulted in increased invasive ability of these cells and increased matrix degradation capability [345]. On the contrary, expression of CD63 in a CD63-negative melanoma cell line reduced cell motility and metastatic capacity [346], likely mediated by the interaction of CD63 with the Integrin  $\beta$ 1. As mentioned, together with TIMP-1 and Integrin  $\beta$ 1, CD63 promote the survival of melanoma cells [321]. In addition exosomes isolated from plasma of melanoma patients contain high levels of CD63 and caveolin-1 [347]. Finally, similarly to melanoma, a decrease of CD63 expression has been correlated to increased malignancy in several tumors [348]–[350].

#### 4. Cholesterol and its intracellular trafficking

Up to this point, I have mostly described the intracellular pathways required for the trafficking of proteins. In this section I will focus on the trafficking and role of lipids and in particular of cholesterol.

Cholesterol is an important membrane component of eukaryotic membranes that contributes to membrane organization and fluidity. Especially by creating liquid ordered domains into membranes, cholesterol participates in several membrane trafficking events and signaling processes. Moreover, dysregulation in cholesterol synthesis or trafficking have been implicated in several pathologies such as cardiovascular diseases, neurodegenerative diseases, cancer and genetic diseases.

Cholesterol contains a hydroxy group as the sole polar group, four hydrocarbon rings that constitute the steroid group and a short alkyl chain. On the contrary, phospholipids are composed by a large polar head group and two long hydrocarbon chains. Because of its structure, cholesterol can easily be absorbed and extracted from membranes. In membranes sterols associate preferentially with phospholipids with bigger polar heads (e.g. phosphatidylcholine and sphingomyelin) and with saturated lipids (e.g. dipalmitoylphosphatidylcholine) [351]. Hence, the different phospholipid composition of cellular membranes can contribute to their enrichment in cholesterol. Indeed, cholesterol appears to be unevenly distributed between cellular membranes (Figure 16) with about 60%-90% of cholesterol being in the PM and only 0.5%-1% being in the ER. Intermediate amounts are present in other organelles in particular in endosomes, with higher amounts in recycling endosomes and on ILVs [182], and in the Golgi with an enrichment in the TGN [351].

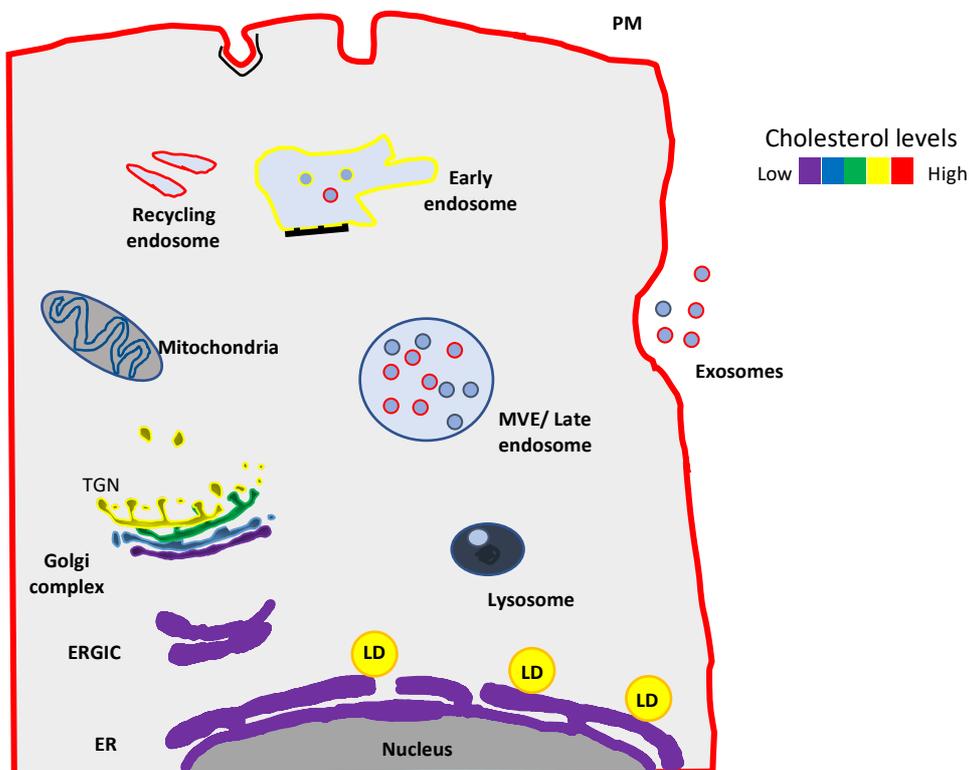


Figure 16. Cellular distribution of cholesterol.

To maintain this abundance and distribution cells have developed different mechanisms that will be briefly described in this section.

#### **4.1. Biosynthesis of cholesterol and regulation of cholesterol metabolism**

In vivo, cholesterol is derived from two sources: de novo synthesis and diet. De novo synthesis occurs in the ER, where key enzymes such as HMG-CoAR ((3-hydroxy-3-methylglutaryl)-CoA reductase) localize [352] with cholesterol sensing proteins involved in the regulation of cholesterol metabolism. Among them, the sterol regulatory element binding protein (SREBP) is a membrane-bound transcription factor that after translocation in the nucleus regulates the transcription of many genes involved in cholesterol metabolism, including HMG-CoAR and low density lipoprotein receptor (LDLR) [353]. When ER cholesterol is abundant, SREBP is associated with its escort protein SCAP (SREBP-cleavage-activating protein) and the ER retention proteins Insig-1 or -2. If cholesterol content in ER is depleted, SCAP undergo a conformational change, the complex SREBP-SCAP is released by Insig and is transported to the Golgi via COPII vesicles [354]. In the Golgi, SREBP is proteolytically processed to generate the transcription factor that will then translocate in the nucleus [355]. An additional level of regulation is given by lanosterol, a precursor of cholesterol, that control the proteosomal degradation of HMG-CoAR [352].

Newly synthesized cholesterol is rapidly removed from the ER, in order to keep ER cholesterol levels low, and transferred to the PM or other organelles. Cholesterol can also be further processed and undergo hydroxylation, to generate oxysterols (e.g. 24,25 or 27-hydroxycholesterol) or fatty acylation, to form cholesteryl esters. Oxysterols are more hydrophilic than cholesterol, therefore they are more easily excreted from cells and more stable in the cytoplasm, where they can act as signaling lipids. On the contrary, cholesteryl esters are more hydrophobic and do not partition in membrane bilayers, but they intercalate into lipid droplets. Esterification occurs in the ER and is regulated by the enzyme acyl-CoA:cholesterol acyltransferase (ACAT), that in presence of high levels of cholesterol in the ER converts cholesterol in cholesteryl esters for their storage in lipid droplets. This cholesterol will be then used by the cell to maintain sterol homeostasis when cellular cholesterol levels are low [356].

#### **4.2. Cholesterol uptake from exogenous sources**

In vivo, cholesterol obtained from the diet is first transported to the liver and then redistributed to other tissues. The transport of cholesterol through the blood circulation is mediated by lipoproteins. Briefly, cholesterol absorbed by enterocytes is packed together with triglyceride to form chylomicrons that are released in the blood circulation. Here some triglycerides are hydrolyzed and apoproteins, such as ApoE, are added up to the generation of chylomicron remnants, which are internalized by hepatocytes. Hepatocytes release lipids through their packaging in very low-density lipoproteins (VLDL). In the blood circulation VLDL are processed to generate intermediate density lipoprotein (IDL) and low-density lipoproteins (LDL) that are then transported and taken up by peripheral tissues. Finally, when

peripheral tissues have excess of cholesterol, they can secrete it in the form of high-density lipoproteins (HDL) that are then delivered to the liver. Chylomicrons, VLDL, LDL and HDL differ for the amount of cholesterol and triglycerides they contain and for their apolipoprotein composition (Figure 17) [357]. LDL represent the main source of cholesterol for peripheral cells. Internalization of LDL occurs through receptor-mediated clathrin-dependent endocytosis. The main group of LDL receptors is represented but the low-density lipoprotein receptor family (LDLR). This family comprises the patriarch of the family LDLR, the VLDLR, the LDL-related protein (LRP1, LRP2, LRP6 and LRP8, also called ApoER2) [358]. A more recent discovered family of receptors is the Vps10p domain receptors that include sortilin and SORLA [359]. These receptors bind to ApoE and ApoB and therefore they participate in the uptake of ApoE and ApoB containing lipoproteins. Once internalized, LDL ends in endosomes where cholesteryl esters are hydrolyzed to generate unesterified cholesterol, that is exported and transferred to other cellular compartments to fulfill cell needs (Figure 17). Export of cholesterol from endosomes likely takes place in early or late endosomal compartments, as suggested by the fact that most of the cholesterol in the endosomal system is found in recycling endosomes and MVEs while very low cholesterol is found in lysosomes.

Lipoprotein	Density (g/ml)	Size (nm)	Major Lipids	Major Apoproteins
Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
Chylomicron Remnants	0.930- 1.006	30-80	Triglycerides Cholesterol	Apo B-48, Apo E
VLDL	0.930- 1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
IDL	1.006- 1.019	25-35	Triglycerides Cholesterol	Apo B-100, Apo E, Apo C
LDL	1.019- 1.063	18- 25	Cholesterol	Apo B-100
HDL	1.063- 1.210	5- 12	Cholesterol Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E

**Figure 17. Classes of lipoproteins.** (adapted from Feingold et al. 2000).

### 4.3. Cholesterol trafficking in endosomes

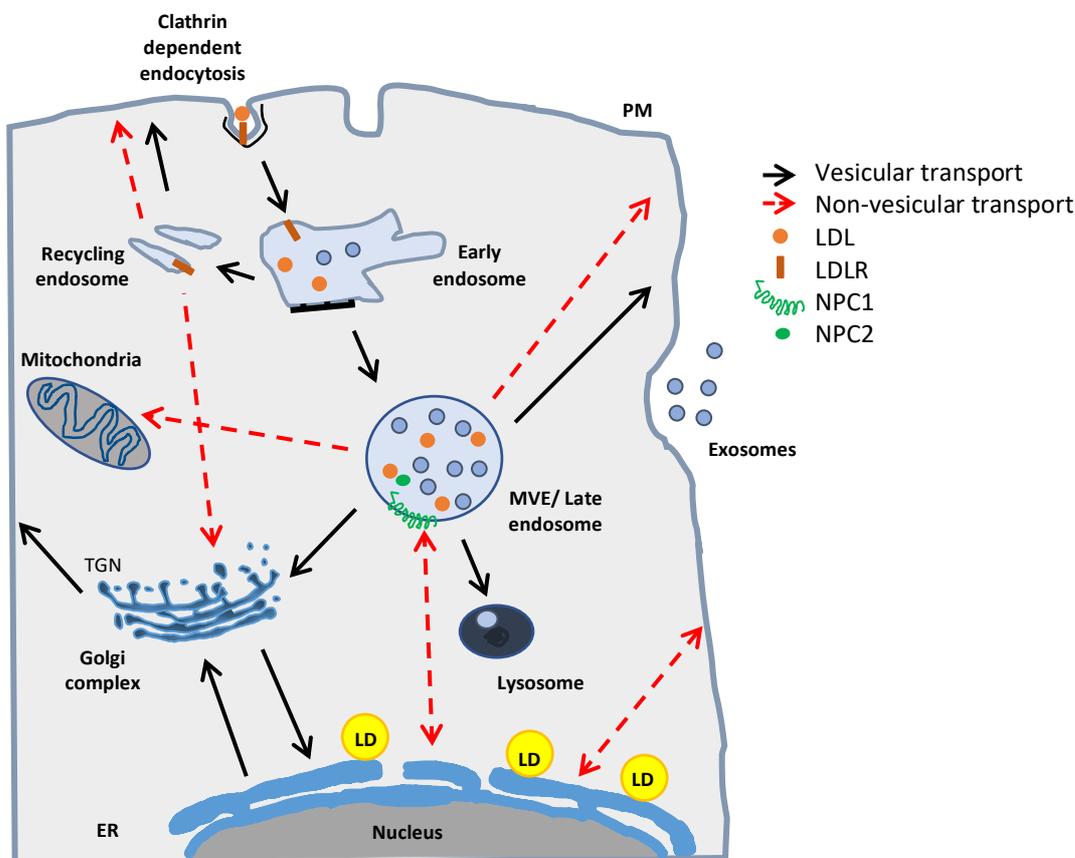
In order to be transferred to other organelles, cholesterol from lipoproteins has to be first transferred to the delimiting membrane of the endosomes. This process is mediated by the subsequent action of two proteins: NPC1 and NPC2, mutations of which cause the Niemann-Pick type C (NPC) disease, a lysosomal storage disease characterized by the accumulation of

cholesterol in late endosomes/lysosomes. NPC1 is a transmembrane protein with 13 transmembrane domains while NPC2 is a small luminal protein. NPC2 is believed to bind free cholesterol in the lumen of endosomes and to transfer it to NPC1 that will finally mediate cholesterol egress from endosomes. Indeed, both NPC1 and NPC2 contain a cholesterol binding domain. NPC1 cholesterol binding domain is situated at the N-terminal of the protein, in the first luminal loop [360]. In addition the region between the third and seventh transmembrane domains represent a sterol sensing domain (SSD) that has been shown to bind a photoactivable analog of cholesterol [361] and to participate in the endosomal sorting of NPC1 [362], while the C-terminus contain a dileucine motif recognized by the adaptor protein AP-1 for the endosomal sorting of NPC1 [363]. Finally residues situated in the second luminal loop of NPC1 mediate its interaction with NPC2 [364]. NPC2 contains a hydrophobic pocket that binds cholesterol in an orientation opposite to NPC1, corroborating the idea that these two proteins act in a sequential manner [365]. Studies from the Goldstein's laboratory identified amino acid residues in both NPC1 and NPC2 that are essential for cholesterol transfer, therefore they propose a model in which these two domains would interact creating a hydrophobic core in NPC1 structure that allows the transfer of cholesterol from NPC2 to NPC1 without passing through the water phase [366], [367]. In vitro studies of the capacity of NPC2 to transfer cholesterol to lipid membranes suggested that the transfer would occur by direct binding of NPC2 to membranes [368]. Hence, it is also possible that NPC2 transfers cholesterol directly to the delimiting membrane of the endosome. From there cholesterol can be transferred to other cholesterol binding proteins (like NPC1) or flip in the cytosolic membrane leaflet and become accessible for transfer to other cellular compartments [369]. Moreover, the capacity of NPC2 to transfer cholesterol to liposomes was significantly increased by specific membrane compositions, in particular by the presence of LBPA [368], a lipid uniquely enriched in internal membrane of late endosomes [184]. This observation suggests that NPC2 can also transfer cholesterol to endosome internal membranes. Interestingly, LBPA together with its interacting protein ALIX, a protein involved in the biogenesis of ILVs, has been shown to regulate endosomal cholesterol levels [300]. In addition, increasing LBPA endosomal levels partially restores the cholesterol accumulation in NPC1 mutant cells [300], [370]. This study proposes a model in which LBPA and ALIX would buffer endosomal cholesterol content by maintaining cholesterol in internal endosomal membranes and by redistributing cholesterol to the delimiting membrane of the endosomes, likely through a back-fusion process. Overall, LBPA could contribute to facilitate cholesterol transport but how its action correlates with NPC2 or NPC1 is still not known. As described before, late endosomes content, including its internal membranes, is degraded after fusion with lysosomes. Hence, these membranes would represent an additional source from which cholesterol can be extracted by NPC2. As shown using an in vitro system, the presence of LBPA or ceramide in the membrane stimulated cholesterol transfer by NPC2, while sphingomyelin inhibited it [371], suggesting that the cholesterol transfer capacity is influenced by the composition of these membranes. Of note, the bidirectional transfer of cholesterol between NPC1 and liposomes is increased by the presence of NPC2, suggesting

that NPC2 could both deliver and remove cholesterol from NPC1 [372]. In conclusion, the precise sequence of events involving NPC2 and NPC1, that ends with the localization of cholesterol in the delimiting membrane of the endosomes, is yet not completely understood.

#### 4.4. Cholesterol egress from endosomes

Once in the delimiting membrane of endosomes, cholesterol need to egress the endosomal system and be transported to other compartments, mostly PM and ER. In general, this process occurs through the formation of vesicular carrier (vesicular transport) or though the formation of membrane contact site (MCS) ([Figure 18](#)).



**Figure 18. Intracellular trafficking of cholesterol.**

Cholesterol is trafficked between membranes through vesicular or non-vesicular transport. Newly synthesized cholesterol exits the ER and is transferred to other cellular compartments. LDL particles containing cholesterol are internalized by clathrin dependent endocytosis of LDL receptors. While empty LDL receptor is recycled back to the PM, free cholesterol is extracted from LDL and transferred to the delimiting membrane of late endosomes by NPC1 and NPC2. From there cholesterol is transferred to other cellular compartments by different mechanisms.

Beside NPC1, several proteins have been involved in the egress of cholesterol from endosomes. Among them we have resident LE/lysosome protein, such as LAMP2 and LIMP-

2, the ESCRT-0 component HRS and the ESCRT-III component VPS4. LAMP2 binds cholesterol and interact with NPC1 and NPC2 [373], in addition LAMP2 alleviate the cholesterol accumulation observed in LAMP deficient cells or after use of the NPC1 inhibitor U18666A [373], [374], suggesting that LAMP2 is involved in the exit of cholesterol from endosomes, probably by storing cholesterol at the delimiting membrane before its transfer to NPC1 via NPC2. A recent study identified LIMP-2 as a new mediator of cholesterol egress from late endosomes/lysosomes. LIMP-2 present a binding cavity for cholesterol. LIMP-2 knockout cells display accumulation of cholesterol in lysosomes while LIMP-2 overexpression alleviate cholesterol accumulation in NPC1 mutant cells, suggesting that LIMP-2 may mediate the egress of cholesterol independently of NPC1 and alongside the NPC2-NPC1 pathway [375]. In the case of HRS, it has been shown that its depletion induces accumulation of LDL derived cholesterol in endosomes and that HRS is required for cholesterol transport to the ER [376]. Despite the precise mechanisms need to be elucidated, the role of HRS may be related to his role in ILV formation, to its role in retrograde trafficking, or to its interaction with ORP5, another protein involved in cholesterol egress from endosomes. Similarly, the same group observed cholesterol endosomal accumulation upon VPS4 depletion and that the role of VPS4 seems to be independent on its role in the ESCRT complex [377].

Different RabGTPases have been involved in cholesterol trafficking. Among them, Rab8 is involved in the egress of LDL derived cholesterol from CD63 positive late endosomes. These transport carriers move along the actin cytoskeleton in a Rab8 and myosin V dependent manner toward the PM, in particular to the leading edge of migrating cells [378]. Moreover, Rab9 activity seem to be impaired in NPC mutant cells [379]. Rab9 overexpression is able to rescue accumulation of cholesterol in NPC mutant cells [379], [380] while its depletion induced cholesterol accumulation in late endosomes/lysosomes, despite only in some cell types [378, p. 8], [379]. Given that Rab9 is involved in vesicular trafficking between endosomes and TGN, it is likely that Rab9 mediates the transport of cholesterol to the TGN, especially in presence of an excess of cholesterol as in the case of NPC mutant cells. Finally, Rab11 overexpression induced accumulation of cholesterol in Rab11 positive recycling endosomes and affect cholesterol esterification, probably due to a defect in the recycling of cholesterol toward the ER [381, p. 11]. Moreover, a recent study identified a new Rab11 effector protein, RELCH/KIAA1468, that seem to tether RE and TGN membranes to allow the non-vesicular transfer of cholesterol to the TGN, probably prior to its trafficking to the ER [382]. As described, Golgi apparatus contain increasing levels of cholesterol going from the cis-face to the TGN. Moreover, NPC mutation or use of U18666A deplete cholesterol in Golgi membranes [383], [384], suggesting that cholesterol could move from LEs/LYs to the TGN prior to its transfer to the ER. Indeed, chasing experiments using radioactive cholesterol showed the appearance of cholesterol in TGN prior to its arrival in the ER. This vesicular transport from LEs to TGN required a functional NPC1 and TGN-specific SNAREs [385].

Annexin proteins have also been involved in the maintenance of cholesterol homeostasis. Annexins are a family of Ca<sup>2+</sup>-binding proteins that participate in membrane traffic and tethering, microdomain organization, cytoskeleton interactions, Ca<sup>2+</sup> signalling and

endosomal homeostasis [386]. Annexin recruitment is influenced by the membrane lipid composition, including the presence of cholesterol. As mentioned above, this is the case of AnnexinA2, which participates in MVE maturation [387]. In the case of AnnexinA6, its overexpression has been shown to induce cholesterol accumulation in endosomes, inducing a NPC1-like phenotype [388]. Hence, AnnexinA6 seem to regulate LE cholesterol transport through a mechanism that remain unknown, but that may involve protein-protein interaction or the formation of membrane contact sites. In this context, another Annexin, AnnexinA1 is involved in the formation of MCS between ER and MVEs, although in this case cholesterol is transferred from the ER to the MVEs [389]. Finally, depletion of AnnexinA8 has been shown to induce cholesterol accumulation in LE/LYs while AnnexinA8 endosomal recruitment is enhanced in cells with endosomal cholesterol accumulation induced by U18666A drug [390].

Non-vesicular transfer of cholesterol requires the binding of cholesterol to carrier sterol transfer protein that places cholesterol in an hydrophobic core and transfers it to membranes that are closely juxtaposed by the formation of membrane contact sites. In MCS the close proximity of membranes of two different organelles allows proteins on one organelle to interact with proteins on the other organelle without having membrane fusion. These contacts are mostly mediated by proteins of the START [STAR (steroidogenic acute regulatory protein)-related lipid transfer] domain family and the OSBP (oxysterol-binding protein) and ORP (OSBP-related protein) families. In the case of endosomes, MCS with the ER represent the faster way for cholesterol transfer to the ER. Principal components of ER-endosomes MCS are the proteins ORP1L, STARD3 (also called MLN64) and ORP5, which are all cholesterol binding proteins. Although these contacts contribute to cholesterol trafficking, they are also involved in the regulation of endosome homeostasis and motility. ORP5, an ER anchored protein, seem to cooperate with NPC1 to regulate cholesterol egress from endosomes. ORP5 knockdown induces accumulation of cholesterol in LE, reducing its transport to the ER and its esterification. In addition, ORP5 interact with NPC1; this interaction may contribute to the formation of MCS, despite experimental evidences are still lacking [391]. ORP1L is a soluble protein and a Rab7 effector that can form a complex with Rab7 and its effector RILP on endosomal membranes. In these complex RILP mediates the interaction with the p150<sup>Glued</sup> subunit of the dynein/dynactin motor complex and promotes the transport of LE toward the perinuclear region. The interaction RILP- p150<sup>Glued</sup> is regulated by the cholesterol sensor ORP1L through the formation of MCS with the ER resident protein VAMP (vesicle associated membrane protein) - associated ER protein (VAP), in a cholesterol dependent manner. When cholesterol levels in endosomes are low, the cholesterol sensing domain of ORP1L changes its conformation allowing the interaction of ORP1L with VAP-A and the formation of MCS, this displaces the interaction with p150<sup>Glued</sup> therefore preventing the transport of LE to the perinuclear region. In case of high cholesterol levels, ORP1L assumes a different conformation that allows its interaction with Rab7/ RILP/ p150<sup>Glued</sup> and the HOPS complex promoting efficient transport of endosomes and fusion with lysosomes [272], [273]. In addition to this role, ORP1L-VAP mediated MCS regulate the egress of

cholesterol from endosomes. In particular, knockout of ORP1L induced accumulation of cholesterol in endosomes, reduction of ER cholesterol esterification and perinuclear redistribution of LE, similarly to NPC1 knockout cells, while the formation of ORP1L-VAP MCS, in presence of functional NPC1 and PI4P allows the transfer of cholesterol to the ER and its further esterification [392]. Moreover, ORP1L-VAP mediated MCS, together with annexinA1 contribute to the transfer of cholesterol from ER to endosomes to sustain the formation of EGFR positive ILVs when LDL-cholesterol levels in endosomes are low [389]. Finally, STARD3 is a sterol binding protein that, at least in some cell types, is localized to a subpopulation of LE distinct from the ORP1L positive subpopulation [393]. STARD3 creates MCS with ER, through its interaction with VAP, mediating the delivering of cholesterol from the ER to late endosomes [394], [395, p. 3]. Of note, another protein called STARD3 N-terminal like (STARD3NL), which also contain a cholesterol binding domain but not a cholesterol transfer domain, may contribute to the formation of MCS [394]. By interacting with STARD3, STARD3NL may create STARD3/cholesterol enriched microdomains on the delimiting membrane of endosomes that may serve to concentrate cholesterol or as sensing platforms [396, p. 3].

#### **4.5. Role of cholesterol in endosomal homeostasis**

The maintenance of cholesterol homeostasis in the endosomal system is of fundamental importance for endosome functioning and positioning.

As mentioned above, endosomal cholesterol content contribute to the positioning of late endosomes and their fusion with lysosomes through ORP1L [272], [273]. In pathological condition such as NPC1 mutation (or inhibition using U18666A) that leads to endosomal accumulation of cholesterol, this pathway is thought to cluster LE in the perinuclear region. Similarly, STARD3 depletion, that would prevent transfer of cholesterol from ER to endosomes has also been shown to promote endosome dispersion [397].

Moreover, endosomal tubulovesicular trafficking is impaired in NPC1 disease [398]. This has implications on the trafficking of protein such as CI-M6PR, which is depleted from the TGN and accumulated in MVEs upon U18666A treatment [399] and whose exit from MVEs requires normal cholesterol endosomal levels [400]. Similarly, recycling of STxB, a cargo of the retromer complex, is impaired after cholesterol extraction [401]. This suggests a potential role of cholesterol in retrieval processes from endosomes that remains to be demonstrated.

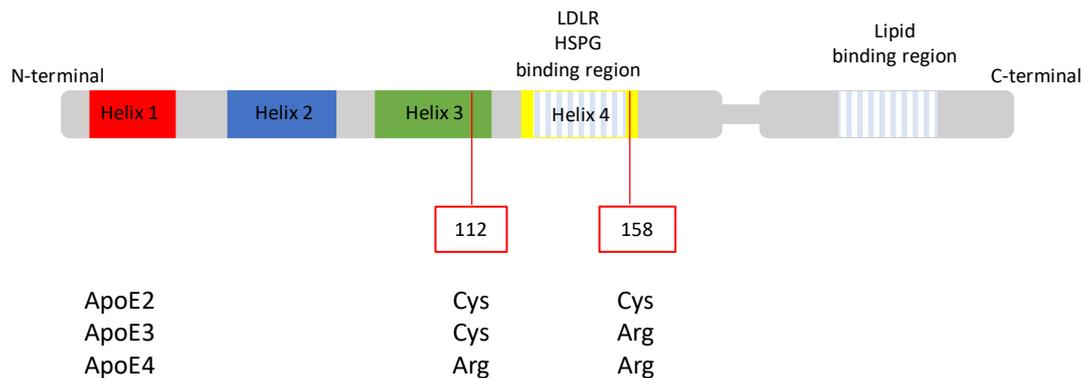
In the context of AnnexinA6-induced cholesterol accumulation, cargo transport to several locations is impaired. This is the case for caveolin-1 that is sequestered in the Golgi and as a consequence it is less present in the PM, or for SNARE proteins. Considering that several SNAREs contain a cholesterol-binding domain and therefore interact with cholesterol, the trafficking of SNARE proteins is indeed dependent on cholesterol levels and localization [402]. Finally, cholesterol-dependent localization and function of SNAREs in turn regulate the delivery of ECM components and integrins to the PM, hence regulating cell migration [402].

## 5. Apolipoprotein E

In this section I will describe the properties and roles of Apolipoprotein E, whose trafficking and roles were investigated during my thesis.

### 5.1. Structure and properties

Apolipoprotein E (ApoE) is a 34 KDa glycosylated protein of 299 amino acids formed by two main structural domains connected by a hinge region [403] ([Figure19](#)).

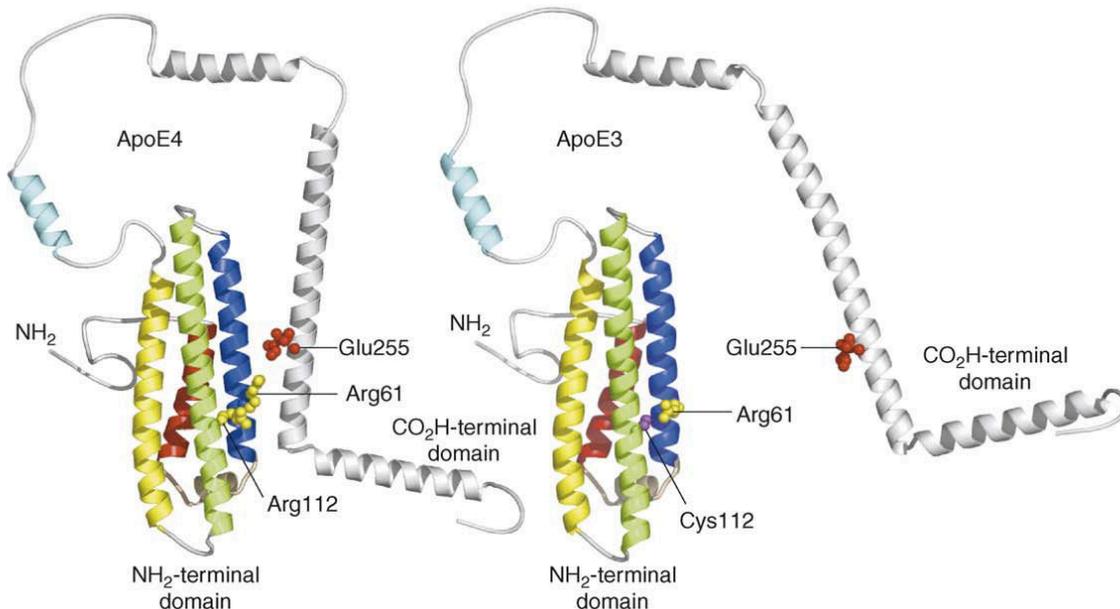


**Figure 19. Linear diagram of Apolipoprotein E structure.**

The N-terminal and the C-terminal domains are connected by a hinge region. The N-terminal contain the binding region for LDLR and HSPGs, while the C-terminal region contain a lipid binding region. Isoform specific sequence differences are indicated.

The amino-terminal region contains the binding domain for the low-density lipoprotein receptor (LDLR) (amino acid 136-150) and a binding domain for heparan sulphate proteoglycans (HSPGs), while the carboxyl-terminal domain contains the lipid binding region. The structure of the amino-terminal region of ApoE, resolved by crystallography, consist of four helices organized in an antiparallel fashion. Although the crystal structure has not been determined yet, the carboxyl-terminal domain consists of amphipathic  $\alpha$ -helices that can bind to lipids. ApoE exist in three isoforms: ApoE2, ApoE3 and ApoE4, which differ for one or two amino acid in the amino-terminal region [404]. Specifically, while ApoE2 contain two cysteine residues in position 112 and 158 and ApoE4 contains two arginine residues in the same positions, ApoE3 present a cysteine in position 112 and an arginine in position 158 ([Figure19](#)). These small differences have implications in the tridimensional structure of the protein ([Figure 20](#)) and influence the receptor binding capacities. For instance, ApoE2 bind LDLR with much lower affinity compared to ApoE3 [405]. Structural studies revealed the presence of a salt bridge in ApoE3 involving the Arg158 that is absent in ApoE2, while in ApoE2 another salt bridge is formed with Arg150, hence eliminating the availability of Arg150 for LDLR binding. The Arg112 in ApoE4 causes an amino acid side chain reorientation that promotes the formation of a salt bridge between the amino-terminal domain and the

carboxyl-terminal domain of the protein, inducing a more compact structure of ApoE4 when compared to ApoE2 or ApoE3.



**Figure 20. Model of lipid-free Apolipoprotein E structure.**

The structure of ApoE4 and ApoE3 is shown. Note the interaction between the N-terminal domain and the C-terminal domain in ApoE4 that is not present in ApoE3 (or ApoE2). (from Hatters et al, 2006).

As mentioned, the carboxyl-terminal domain of ApoE contain a binding domain for lipids. ApoE binds with micromolar affinity lipid emulsions containing a triglyceride core surrounded by a phospholipid monolayer, a structure which is reminiscent of VLDLs. Moreover, when added to phospholipid vesicles, ApoE reorganizes the lipids to forms small discoidal or spheroidal ApoE-phospholipid complexes, that are reminiscent of HDL. Despite the tridimensional structure of lipid-bound ApoE has not been determined, it is likely to differ from the lipid-free structure and may also vary according to the type of lipids to which ApoE is bound [403]. So far, different models have been proposed and all involve the carboxyl-terminal domain of ApoE [403]. For instance, ApoE could interact with the acyl chains of a discoidal bilayer of phospholipids through its  $\alpha$ -helices assuming a “belt-like” configuration. Other studies suggest that ApoE do not form a complete belt but that it interacts either with the edge of the lipid bilayer or with the phospholipid headgroups in elliptical and discoidal particles. Finally, ApoE can interact with the phospholipid polar headgroups of a spheroidal lipoprotein. In this case only two molecules of ApoE are associated to the lipoprotein and they do not form a complete belt around the particles but are staggered at a  $42^\circ$  angle to each other.

The different ApoE isoforms differ also for the conformational stability of their amino-terminal region, with ApoE4 being the less resistant to denaturation and ApoE2 being the most resistant. Contrarily, ApoE2 amino-terminal domain unfolds directly from a native to an

unfolded state, while ApoE3, and even more ApoE4, can also form intermediate stable conformations that can facilitate the conformational changes required for ApoE lipid binding.

The structural differences between the “wild type” ApoE3 and the other two isoforms have implications in pathological conditions, with ApoE2 being correlated with Type III hyperlipoproteinemia and ApoE4 being correlated to higher risk and earlier onset of Alzheimer’s disease but also with other neurodegenerative diseases such as Parkinson’s disease, multiple sclerosis, dementia with Lewy bodies and tauopathies. In addition, people homozygotes for ApoE4 have lower levels of ApoE in plasma but higher levels of cholesterol and LDL. This phenotype may be explained by a higher clearance of ApoE4-containing particles [406] from the plasma and by the preference of ApoE4 to bind to larger lipoproteins such as VLDL and chylomicrons [407] that will be then converted into LDL. Furthermore, the domain interaction in ApoE4 and the formation of partially folded intermediates can contribute to the generation of proteolytic fragments of ApoE that may be involved in the pathogenesis of Alzheimer’s disease [408], [409].

Finally, ApoE is post-translationally glycosylated [410]. In humans, ApoE is exclusively O-glycosylated and may be capped with a sialic acid, a negatively charged carbohydrate. The extent of glycosylation and sialylation is very heterogenous and cell-type specific. ApoE secreted by hepatocytes is highly glycosylated and sialylated but ApoE in the plasma is not. This would suggest that at least a part of the sugars is removed by glycosidases in the blood circulation. On the contrary, ApoE in the cerebrospinal fluid (CSF) remain highly glycosylated and sialylated after its secretion by astrocytes. A single glycosylation site at threonine-194 was at first identified for ApoE in the plasma and secreted by cells [411]. ApoE secreted by human macrophages is also O-glycosylated on Ser290 in the carboxyl-terminal domain. In addition to these two sites, ApoE in the CSF is glycosylated on Thr8, Thr18, Ser296 and S289. Glycosylation is dispensable for ApoE translation and secretion, but it may regulate specific ApoE functions. For instance, the presence of sialic acid at Thr194 in the CSF seem to enhance the association of ApoE with the toxic amyloid  $\beta$  1-42 peptide, suggesting that glycosylation may affect the formation of amyloid plaques [412].

## 5.2. ApoE secretion

ApoE is secreted from many cells throughout the body, including hepatocytes, macrophages, adipocytes, melanocytes and cells of the central nervous system. Hepatocytes are the main producers of ApoE in the body and most of the ApoE found in the plasma is derived from the liver. In hepatocytes, newly synthesized ApoE associates with nascent VLDL in the Golgi apparatus before being secreted. ApoE secretion is also stimulated by the acquisition of lipoproteins (e.g. HDL) by the cell. In addition, lipid-free ApoE can be secreted, remaining in large part attached to HSPG on the cell surface or self-associating into tetramers [413].

In the blood circulation ApoE associates to different class of lipoproteins, in particular chylomicrons, chylomicron remnants, VLDL, IDL and a subgroup of HDL particles, and can be exchanged in between lipoproteins (Figure 17). In peripheric tissues, the presence of ApoE

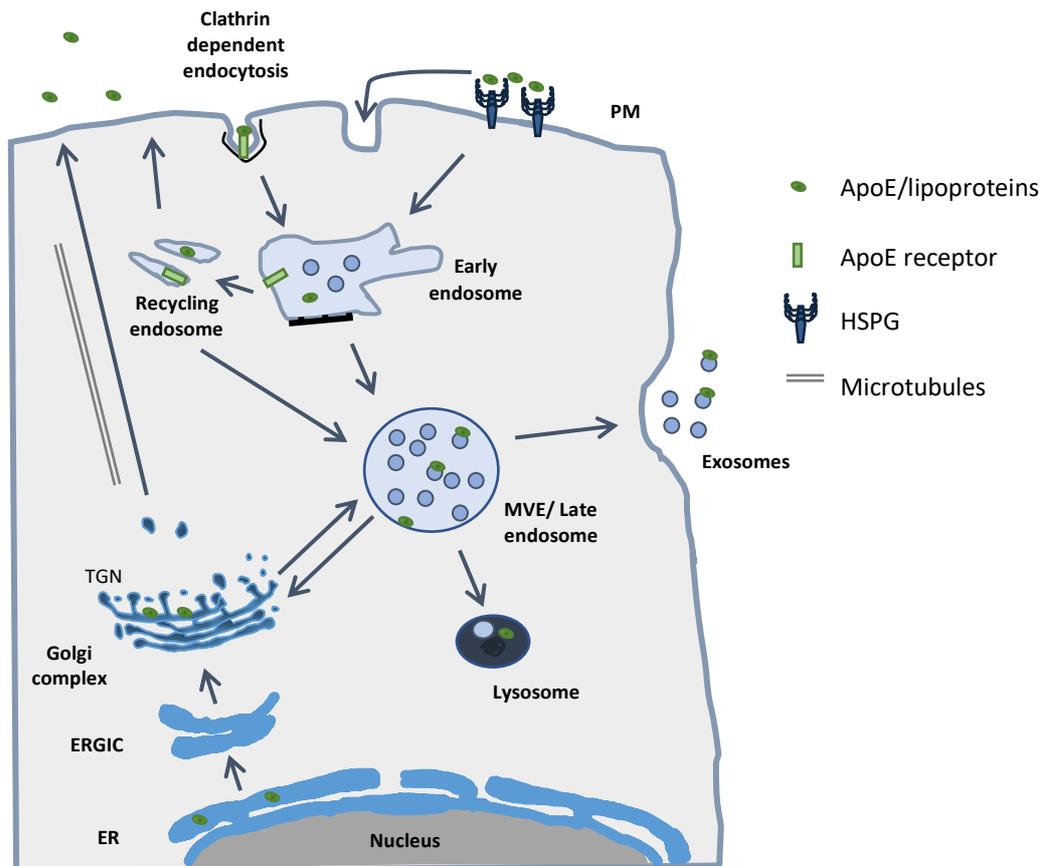
on VLDL mediates their uptake by adipose tissue, muscle cells and heart to deliver triglycerides for energy or storage. The presence of ApoE on HDL contributes to HDL uptake through binding to LDLR, LRP1 and HSPGs in the liver. Hence, ApoE mediates both cholesterol transport from the liver to peripheral tissues and reverse cholesterol transport from peripheral tissues to the liver.

Local tissue-secretion of ApoE has also important roles [410]. For instance, macrophage derived ApoE protects against atherosclerosis and has anti-inflammatory, anti-proliferative and immune-modulatory properties. In mice ApoE has been shown to regulate monocyte differentiation in macrophages and to regulate the transition from pro-inflammatory M1 to anti-inflammatory M2 macrophages, but it is still unknown if such functions are also executed in humans. Endogenous adipocyte ApoE have a role in regulating cell size, triglyceride content and inflammation of the adipose tissue. In the brain ApoE is secreted mainly by astrocytes and to a lesser extent by neurons and microglia. ApoE is the main apolipoprotein responsible for lipid transport in the central nervous system, mostly for the delivery of cholesterol and phospholipids from astrocytes to neurons. The expression of ApoE in astrocytes, neurons and microglia is increased after brain injury suggesting that ApoE may have a role in neuroprotection and repair [414]. Interestingly, while all other peripheral tissues mostly receive cholesterol from liver-derived lipoproteins, the majority of cholesterol in the brain is synthesized in situ and transported by specific lipoproteins containing ApoE, A1, D and J but not ApoB and resembling plasma HDL. The majority of these HDL-like particles are released by astrocytes and contain ApoE. Similarly to the formation of HDL in the plasma, it is believed that lipid-free ApoE associates to lipids extracellularly through the action of the ABC transporters, which mediates the efflux of lipids and cholesterol from cells. The nascent ApoE-containing lipoproteins are discoidal and contain little lipid primarily unesterified cholesterol and phospholipids with no cholesteryl esters [415].

In addition to lipoproteins, ApoE can be secreted through exosomes. Exosomal release of ApoE has been shown in different cell types including melanocytes, brain cells, macrophages and hepatocytes [140], [416]–[419]. Despite the intracellular pathways contributing to ApoE secretion on exosomes have not been elucidated yet, the study I conducted during my thesis shows that ApoE is a CD63-dependent and ESCRT-independent cargo. In addition, the proportion of ApoE that is secreted through exosomes or through lipoproteins has not been determined yet and may greatly vary in between cell types. Nevertheless, ApoE-exosomes seem to play a role in neurodegenerative diseases [418] and cancer [419].

### **5.3. Intracellular trafficking of ApoE**

The intracellular trafficking of ApoE has been so far mostly studied in macrophages and hepatocytes. ApoE is a secreted protein that mostly follow the conventional secretory pathway for its secretion ([Figure 21](#)).



**Figure 21. Intracellular trafficking of ApoE.**

ApoE is secreted through Golgi-derived vesicular carriers that move along microtubules. Lipid-free ApoE can remain bound to HSPGs on the cell surface. Internalization of ApoE or ApoE-containing lipoproteins occurs through receptor and clathrin-mediated endocytosis. HSPGs can also promote ApoE endocytosis. Internalized ApoE can undergo recycling to the cell surface or to the Golgi apparatus. Alternatively to secretion in the extracellular space, ApoE might be transported directly from Golgi to endosomes. Finally, the endosomal pool of ApoE may be fated for lysosomal degradation or for exosomal secretion.

Early ApoE trafficking from ER to Golgi apparatus is inhibited upon accumulation of free cholesterol in the ER [420]. Once in the Golgi, ApoE is glycosylated and released in the extracellular environment through Golgi-derived vesicles that move along the microtubule network. Secretion of ApoE from macrophages is regulated by protein kinase A-mediated signaling and by intracellular  $\text{Ca}^{2+}$  [421]. Further studies identified a role for protein kinase C and its effector myristoylated alanine-rich protein kinase C substrate (MARCKS) [422] and for the GTPase dynamin II [423]. Although ApoE is constitutively secreted, extracellular stimuli, including exposure to HDL or to phospholipid discs containing ApoA-I, can induce ApoE secretion [424], [425]. More in detail, in macrophages ApoE is synthesized in excess of cellular needs, hence while one pool is fated for secretion, another pool is fated for degradation. Such pool might derive from a direct transport from Golgi to endosomes, but this pathway has never been demonstrated. Extracellular stimuli can redirect the ApoE from

the degradative pathway to the secretory pathway. As mentioned, secreted ApoE can remain bound to HSPGs on the cell surface. Such pool can be internalized and be subsequently degraded, recycled back to the Golgi for further post-translational modification and re-released in the extracellular space via recycling endosomes [410]. Pulse-chase experiments of ApoE-VLDL in Chinese hamster ovary cells revealed that ApoE is recycled through early and recycling endosomes suggesting that this pathway may be linked to cholesterol efflux via recycling endosomes [426]. In addition, ApoE recycling seem to be biologically relevant in vivo, given that hepatocytes from ApoE knockout mice were able to re-secrete macrophage derived ApoE [427]. Mechanistically, recycling of triglyceride-rich lipoprotein-derived ApoE in hepatocytes seem to be regulated by the receptor LRP1, which would enable the accumulation of ApoE in early endosomes prior to its re-secretion in association with HDL [428]. Finally, LRP1 has been involved in the degradation of endocytosed lipid-poor ApoE [429].

#### **5.4. ApoE transport after uptake by receptors**

Two main classes of receptors mediate the uptake of ApoE-containing lipoproteins. As mentioned above, ApoE receptors are members of the LDL receptor family (LDLR, VLDLR, LRP1, LRP2, LRP6 and LRP8) [358] or members of the Vps10p domain receptor family (sortilin and SORLA) [359]. The LDL receptor family members are characterized by the presence of one or more ligand-binding domains, an epidermal growth factor homology domain and a  $\beta$ -propeller domain involved in the PH-sensitive release of ligands in endosomes, a single transmembrane domain and a NPxY motif required for receptor internalization. In addition to ApoE-lipoproteins, LDLR family members bind a variety of extracellular ligands. The receptors exhibit distinct binding affinity for ApoE, depending on ApoE isoforms and its lipidation state. As mentioned, ApoE2 has lower affinity for LDLR and LRP1, when compared with ApoE3 or ApoE4. While LRP1 binds recombinant ApoE or ApoE aggregates, LDLR binds to lipidated ApoE and VLDLR to lipid-free ApoE [430]. LDLR is ubiquitously expressed and is essential for lipoprotein uptake and lipid metabolisms. Genetic mutations impairing LDLR function result in elevated serum LDL levels and early onset of atherosclerosis known as familial hypercholesterolemia [358]. VLDLR is expressed in adipose tissues, skeletal muscle, heart and endothelial cells but not in hepatocytes and it primarily modulates the extra-hepatic metabolism of triglyceride-rich lipoproteins. LRP1 is ubiquitously expressed but particularly abundant in liver and brain. LRP1 is mostly involved in the uptake of ApoE-rich chylomicrons remnants in the liver and in the clearance of VLDL in the liver, muscle cells and macrophages. In addition to ApoE, more than 40 additional LRP1 ligands have been identified, including proteases and protease-inhibitor complexes, coagulation factors, extracellular matrix (ECM) proteins, growth factors and signaling proteins. Moreover, LRP1 can bind other transmembrane receptors, including integrins and receptor tyrosine kinases and modulate their trafficking and signaling [431]. Hence LRP1 participate to many physio-pathological processes including maintenance of vascular integrity, protection against

atherosclerosis, modulation of the blood brain barrier, Alzheimer's disease, cell migration and cancer, immune response [431].

As mentioned earlier in this manuscript, sortilin, a member of the Vps10p family, is a known receptor for lysosomal proteins, that mediate the anterograde transport from TGN to endosomes. Sortilin was first identified in the brain in an attempt to identify new lipoprotein receptors [432]. Sortilin is mainly expressed in neurons of the central and peripheral nervous system, in hepatocytes and white blood cells. In the liver, sortilin seem to contribute to lipoprotein metabolism by facilitating the clearance of LDL from the circulation and by regulating secretion and degradation of VLDL [433]. In the brain, sortilin regulates the intracellular trafficking and functions of neurotrophins, a class of growth factors, and their receptors, hence participating to development, maintenance and function of the nervous system. Moreover, sortilin has been implicated in the development of neurodegenerative diseases, including Alzheimer's disease. Such role is directly linked to sortilin activity as ApoE receptor, given that, in neurons, sortilin represent a main pathway of clearance of toxic amyloid- $\beta$  peptide ( $A\beta$ )/ ApoE complexes [434]. Beside this, sortilin controls  $A\beta$  generation by regulating the trafficking of its precursor APP and its processing enzyme  $\beta$ -secretase [435], [436].

Finally, both lipidated and non-lipidated ApoE bind to cell surface HSPGs [437]. HSPGs can form a complex with LRP1, with the two receptors cooperating for ApoE uptake [438].

## **5.5. Role of ApoE in diseases**

ApoE isoforms have been involved in several pathologies including neurodegenerative diseases, atherosclerosis and cardiovascular risk, cancer. In addition, ApoE contribute to the assembly and secretion of Hepatitis C Virus (HCV), a process that share features with the biogenesis of both lipoproteins and exosomes. Such roles will be briefly described in the following paragraphs.

### **5.5.1. Neurodegenerative diseases**

As previously mentioned, ApoE4 has been correlated to higher risk and earlier onset of Alzheimer's disease (AD) but also to other neurodegenerative diseases. ApoE and in particular ApoE4 seem to modulate several pathways contributing to AD, including metabolism, aggregation and toxicity of  $A\beta$  peptide, lipid and glucose metabolism, mitochondrial function, vascular integrity and neuroinflammation [430]. One of the main features of Alzheimer's disease is the generation and accumulation of toxic amyloidogenic  $A\beta$  peptides originated by the cleavage of APP by the  $\beta$ -secretase BACE-1 and the  $\gamma$ -secretase complex.  $A\beta$  aggregation and cerebral deposition seem to be accelerated in subjects with ApoE4 genotype. On the contrary ApoE2 genotype, being associated with milder  $A\beta$  pathology and slower cognitive decline, seem to have a neuroprotective effect. Recent studies have revealed that ApoE association with  $A\beta$  can stabilize  $A\beta$  seeds and fibrils and slow down the formation of  $A\beta$  fibrils from  $A\beta$  oligomers [439], [440], with ApoE4 being less efficient in stabilizing  $A\beta$  fibrils [441]. Moreover, accumulation of toxic  $A\beta$  results from the

balance between A $\beta$  production and clearance. ApoE and its receptors seem to participate in both these processes. Apart from ApoE association with A $\beta$  fibrils, ApoE receptors influence A $\beta$  production by modulating the intracellular trafficking of APP and its processing enzymes. Moreover, ApoE isoforms can differentially modulate APP transcription, so A $\beta$  production [442, p. 2]. Clearance of A $\beta$  takes place through different pathways, including receptor-mediated uptake, cerebrovascular clearance and proteolytic degradation. Receptor-mediated uptake of A $\beta$  is regulated by different ApoE receptor, including LRP1, LDLR and HSPGs, and can take place in both the brain parenchyma (neurons and glial cells) and the cerebrovasculature [430]. ApoE forms complexes with A $\beta$ , which are in turn internalized through LRP1 or LDLR followed by ApoE recycling and lysosomal degradation of A $\beta$  [443]. Being ApoE4-A $\beta$  complexes less stable, ApoE4 is less effective in promoting A $\beta$  clearance [444]. Alternatively ApoE could compete with A $\beta$  for the binding to common receptors, hence impairing A $\beta$  uptake [445]. Finally proteolytic degradation of A $\beta$  by endopeptidases is also differently modulated by ApoE isoforms [430]. A $\beta$ -independent roles of ApoE also contribute to AD pathogenesis. First, ApoE per se can represent a neurotoxic factor. Several studies showed that, when expressed in neurons, ApoE4 is more susceptible than ApoE3 to proteolytic degradation resulting in the formation of C-terminal fragments. The presence of such fragments have been associated with tau phosphorylation and mitochondrial dysfunction, two other features of AD [446]. Tau, a microtubule associated proteins, represent the main constituent of intracellular neurofibrillary tangles which accumulate in neurons in different neurodegenerative diseases, including AD, known as tauopathies. It has been shown that neuronal ApoE4 induces tau phosphorylation, despite the precise mechanism is still not known [446]. Finally other processes in which ApoE participates during AD development include neuroinflammation, glucose and iron metabolism and maintenance of vascular integrity [446].

### **5.5.2. Atherosclerosis**

Atherosclerosis consists in the narrowing of arteries due to the formation of a plaque, containing fat, cholesterol, calcium and other blood components, which in turn can result in cardiovascular pathology including stroke and artery disease. Atherosclerosis involves both lipid accumulation and inflammatory processes that features macrophages. Expression of ApoE in macrophages has been long considered protective against atherosclerosis and to prevent the formation of foam cells, a type of macrophages that localize to the plaque and are laden with lipids. Such ApoE property is mostly related to the lipid efflux capacities of ApoE. In addition, by interacting with its receptors and HSPGs, ApoE induces cellular signaling pathways that control processes such as macrophage plasticity, smooth muscle cell proliferation and endothelial cell activation [447]. ApoE also regulates inflammatory processes, including proliferation of T cells, activation of endothelial cells and platelets. Another mechanism by which ApoE exert its protective function is the regulation of macrophage polarity, with ApoE promoting the switching between pro-inflammatory M1 and anti-inflammatory M2 macrophages. Finally, recent studies have involved ApoE in the

regulation of the expression of specific miRNAs in macrophages that in turn regulate inflammatory processes [448]. In conclusion, ApoE seem to play a pleiotropic role in atherosclerosis pathogenesis. For this reason, ApoE knockout mice, with or without knock-in of specific ApoE isoforms, are widely used as a model to study atherosclerosis.

### **5.5.3. HCV infection**

Hepatitis C virus (HCV) is an RNA virus that can establish chronic infection in humans. HCV partially hijack the lipoprotein pathway to allow its replication cycle and HCV particle formation, with ApoE being an essential element in this process [449]. As for VLDL, HCV particles form in the ER, with ApoE and other apoproteins being incorporated also at this step. HCV uses the blood circulation for viral transmission to other cells. In the blood HCV is associated to lipoproteins forming lipo-viro-particles (LPVs) which expose ApoE on their surface. Such ApoE can also be lipid- free ApoE which associated extracellularly to HCV-particles mediating the maturation into infectious LPVs. Finally, in order to infect other cells, LPVs are captured on the cell surface by HSPGs and other receptors, including the ApoE receptors LDLR and VLDLR, the HDL scavenger receptor SR-B1, prior entering the cell through interaction with the tetraspanin CD81, claudin-1 and occludin.

### **5.5.4. Cancer**

ApoE expression or functions have been also implicated in the pathogenesis of cancer. First, ApoE isoforms have been correlated to higher risk of cancer, as in the case of ApoE4 for breast cancer or ApoE3 for prostate cancer [450], [451]. ApoE resulted to be overexpressed in human gastric cancer and was correlated to shorter survival and muscular invasion [452]. Similarly, ApoE is overexpressed in colorectal cancer and associated with poor survival [453]. Moreover, elevated levels of ApoE in serum have been associated to a poor prognosis in patients with breast cancer or lung cancer [454], [455]. Contrarily to these reports, ApoE has been identified as a negative regulator of melanoma progression by acting on a variety of pathways. ApoE expression was lower in metastatic cell lines and in patients with metastatic cancer compared to non-metastatic cancer [456], [457]. ApoE expression in melanoma cells is negatively regulated by the expression of endogenous microRNAs (miR-1908, miR-199a-3p, and miR-199a-5p) and inactivation of ApoE promotes metastasis and angiogenesis. Mechanistically ApoE, secreted by melanoma cells, interact with LRP1 on the surface of melanoma cells inhibiting their migration. In addition, extracellular ApoE can engage the receptor LRP8 on endothelial cells, inhibiting their migration, hence suppressing angiogenesis [456]. A following study from the same group showed that induction of the liver-X-receptor (LXR), which control the transcription of ApoE, also suppress melanoma progression by inducing expression of ApoE [457]. Moreover, induction of LXR in vivo induces expression of ApoE in several cell types in the tumor microenvironment, including macrophages, leukocytes and endothelial cells, but also in other tissues [457]. Finally, the LXR/ApoE axis promotes anti-tumor immunity by targeting immunosuppressive immune cells [458]. Although these studies reported the secretion of ApoE by melanoma cells, they

did not investigate the intracellular trafficking and the pathway of secretion of ApoE. Moreover, beside an autocrine action, melanoma derived ApoE may also have a paracrine effect and modulate the tumor microenvironment, including the ECM. Hence, these questions were partially addressed during my thesis.

## 6. Melanoma and role of EVs in cancer progression

In this section I will illustrate the different roles of extracellular vesicles in cancer progression. Moreover, given that melanoma cells and melanoma derived EVs were used in this work to study their capacity of interacting with extracellular matrix components, I will briefly describe the main features of melanoma and the composition of the extracellular matrix.

### 6.1. Melanoma

Melanoma is one of the most aggressive and treatment-resistant cancers, with an overall mortality rate of 20%, mostly due to its high capacity to metastasize in other organs [459]. Melanoma derives from melanocytes localized in the skin, but also in the uvea of the eyes, in the inner ear, meninges, in bones and heart. In the skin, melanocytes are localized in the basal layer of the epidermis and are surrounded by keratinocytes. A basement membrane separates the epidermis from the dermis, which contain nerve endings, blood and lymph vessels, fibroblasts and macrophages surrounded by extracellular matrix. Exposure to UV radiation, with consequent DNA damage, is considered a major etiologic factor in the pathogenesis of these tumors. Primary melanomas are often associated to precursor lesions, including benign nevi and dysplastic nevi that may then evolve to a melanoma in situ and to an invasive melanoma [460]. The transformation of melanocytes into melanoma is a multistep process starting with the horizontal or radial growth due to uncontrolled proliferation of melanocytes. In melanomas, proliferation is mostly regulated by MAP kinase signaling (Ras/Raf/MEK/ERK), which is activated in more than 80% of the cases. Indeed, the mutation of BRAF (BRAFV600E) is the most common genetic change found in melanoma, which is already found in benign and dysplastic nevi. Other mutations, such as loss of function of p53 or p16<sup>INK4a</sup> or overexpression of telomerase reverse transcriptase, contribute to the immortalization of melanocytes, hence favoring the radial growth phase [461]. Invasive phase, also called vertical growth phase, start when melanocytes break the basement membrane and enter in the dermis. During this switch melanoma cells undergo an epithelial to mesenchymal transition (EMT)-like process, in which they lose contacts with the neighboring keratinocytes and acquire migratory and invasive capacities. It is not clear if melanoma cells migrate singularly or collectively, but once in the dermis they can create “nests” of cells that are often observed in histological sections of melanoma lesions. Different pathways of metastatic spread have been described for melanoma [462]. Melanoma cells can enter the blood circulation and be transported to distant organs or they can enter the lymphatic circulation and create metastasis in lymph nodes. From there, melanoma cells can be subsequently transported to distant organs and create metastasis, likely through vascular circulation. Apart from lymph nodes, lung and brain are the main sites of melanoma metastasis [460].

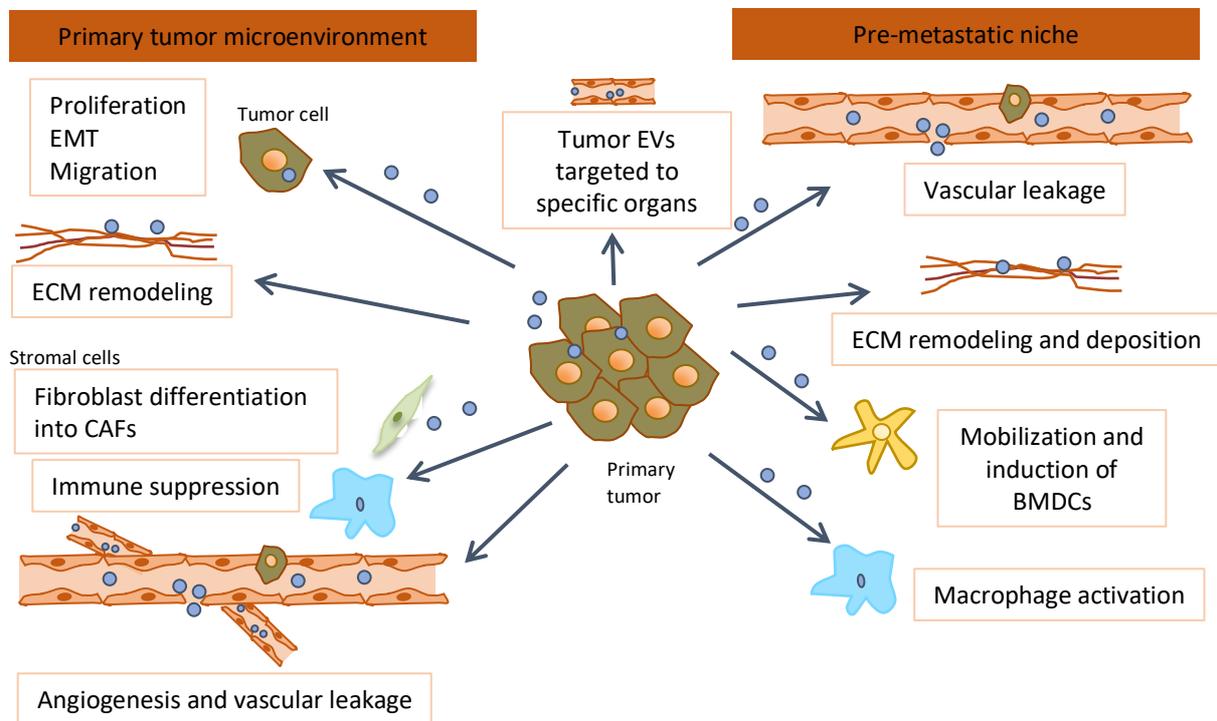
## 6.2. Tumor microenvironment and extracellular matrix

In addition to cell autonomous signaling and tumor cell mutations, tumor microenvironment plays a fundamental role in cancer progression. The tumor microenvironment comprises various cell types (e.g. endothelial cells, fibroblasts, immune cells), other extracellular components (e.g. ECM, cytokines and growth factors) and the vasculature [463]. It is now well known that stromal and immune cells, including fibroblasts and macrophages change their phenotype when present in the tumor microenvironment. For instance, cancer-associated fibroblasts (CAFs) are an activated subpopulation of fibroblasts with higher rate of proliferation and ability to migrate, which secrete and remodel ECM, promote inflammation, angiogenesis and tumor growth. Tumor-associated macrophages (TAMs) are also abundant in the tumor microenvironment and they can inhibit or promote tumorigenesis according to their polarization state. Similarly, tumor endothelial cells (TECs) differ from normal endothelial cells in terms of proliferative and migratory capacities, and response to growth factors. Consequently, the structure of the vasculature within the tumor being abnormal, immune cell extravasation and recruitment to the tumor site is impaired. In addition, angiogenesis plays a crucial role in tumor growth and metastasis and contribute to oxygen and nutrient supply to the tumor. Nevertheless, areas of hypoxia and limited nutrient supply are often found in the tumor and also contribute to tumor cell survival.

Moreover, the extracellular matrix within the tumor microenvironment appeared altered if compared to normal tissues. In general, the ECM is a macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins and other glycoproteins [464]. All cell types secrete ECM molecules under the control of multiple signaling pathways, hence participating to ECM formation. Cells embedded into the ECM interact with ECM components through specific receptors such as integrins, discoidin domain receptor, cell surface proteoglycans and the hyaluronan receptor CD44. Upon interaction, cells integrate signals from the ECM that modulate their functions and behavior. ECM remodeling, occurring both in physiological and pathological conditions, is mediated by a variety of enzymes, including metalloproteases (e.g. MMPs and ADAMs), cathepsins, heparanases and plasminogen activators. In addition, growth factors are sequestered within the ECM via binding with ECM components and are released upon ECM degradation. Hence, in the context of cancer, ECM remodeling would not only contribute to cancer cell migration but also to cancer cell transformation by liberating soluble factors, such as growth factors and cytokines.

Overall, intercellular communication between tumor cells and the different components of tumor microenvironment is essential for cancer progression. Beside soluble factors, tumor- or stroma-derived EVs have been shown to participate to all phases of cancer progression including tumor cell transformation, ECM remodeling, invasion and metastasis establishment ([Figure 22](#)) (see below). Moreover, in melanoma in situ, melanosomes have recently been shown to transfer specific miRNAs to dermal fibroblasts favoring their conversion into CAFs, hence contributing to the formation of a tumor primary niche into the dermis [465].

Finally, stromal cells can themselves release EVs which will in turn modulate tumor cell proliferation, migration and metastatic capacity.



**Figure 22. Role of EVs in cancer progression.**

### 6.3. Role of EVs in the primary tumor microenvironment

Tumor derived EVs can provide autocrine and paracrine signals within the tumor which induce epithelial-mesenchymal transition (EMT), hence promoting invasion of tumor surrounding tissues [466]. Indeed, inducers of EMT, including TGF $\beta$ ,  $\beta$ -catenin, TNF $\alpha$  and others, have been found in EVs. Of note, their secretion can be modulated by specific conditions, including hypoxia. In addition to proteins, miRNA contained in EVs have been implicated in the regulation of EMT. Finally, in case of Epstein-Barr virus (EBV)-mediated cancer pathogenesis, exosomes containing the EBV-derived protein LMP1 seem to contribute to metastatic phenotype by inducing EMT [467].

Alternatively, tumor derived EVs can contribute to the metastatic cascade by acting on the tumor stroma. As mentioned, angiogenesis within the tumor microenvironment is a major hallmark of cancer progression. Tumor EVs can promote angiogenesis by inducing endothelial cell proliferation, migration and vessel branching, as shown for Tspan8 positive EVs [468]. In addition, hypoxia, a major stimulus of angiogenesis, has been shown to promote EV release and to modulate their composition. Such EVs may in turn modulate angiogenesis by transferring their content to endothelial cells or by inducing the release of angiogenesis promoting factors such as VEGF. In a similar manner, tumor EVs can be

transferred to fibroblasts and induce their differentiation into CAFs [469]. Furthermore, tumor derived EVs can modulate immune response in several ways [470]. Tumor EVs contain tumor antigens that can be transmitted to dendritic cells and cross-presented to T-cells to induce tolerance against the tumor. EVs can also modulate the proliferation and survival of immune cells, as shown for melanoma EVs containing Fas-ligand which induce apoptosis of T-cells, after engaging their Fas receptor [471]. Finally EVs containing tumor antigens can modulate immune response indirectly, for instance by sequestering antibodies, hence impairing antibody-dependent cell cytotoxicity [472], a process that may modulate cancer response to therapy, when antibodies are used.

Another important step toward metastasis formation is represented by the migration of tumor cells and by their subsequent intravasation to enter the blood circulation. Several studies have investigated the role of EVs in promoting tumor cell migration, mostly using in vitro assays. In particular, exosomes can promote directional cell motility through ECM components, such as fibronectin, which by interaction with the ECM, would provide a substrate favoring cell adhesion and motility [473]. Moreover, the same group showed that exosome secretion in migrating cells preferentially occurs in proximity of invadopodia and also promote cancer cell invasiveness [202]. Several studies have identified metalloproteases in EVs [474]. Such proteases can contribute to ECM degradation and remodeling, hence favoring cancer invasiveness [475], [476]. Finally, EVs can promote intravasation of cancer cell in the blood circulation. For instance, exosomes secreted by metastatic breast cancer and carrying miR-105 have been shown to destroy tight junctions in endothelial monolayers, hence increasing vascular permeability and promoting metastasis [477].

#### **6.4. Role of EVs in pre-metastatic niche formation**

The final step of the metastatic cascade consists in the arrival of cancer cells in a distant organ where they will proliferate creating a secondary tumor. This process implies the presence of a microenvironment in the distant organ, which is well prepared for tumor cells to colonize and disseminate. Such microenvironment is referred as pre-metastatic niche. Several factors contribute to the formation of a pre-metastatic niche, including tumor-derived secreted factors, tumor-derived EVs, bone marrow derived cells (BMDCs), suppressive immune cells and stromal cells. Homing of cancer cells to specific distant organs is regulated by several factors, including the expression of chemokine receptors that will partner with chemokine ligands expressed in distant organs, the secretion of secreted factors by tumor cells which contribute to establish a favorable microenvironment, and EVs. In particular, circulating EVs from different tumor types bear integrins that target them to specific organs where they will initiate pre-metastatic niche formation. For instance, EVs expressing  $\alpha_v\beta_5$  integrin bind to liver cells, while EVs bearing  $\alpha_6\beta_4$  or  $\alpha_6\beta_1$  bind lung-resident fibroblast and epithelial cells respectively, leading to lung tropism [295]. Once in distant organs, EVs have been shown to contribute to the formation of a pre-metastatic niche in several ways, including BMDCs mobilization, blood brain barrier disruption in case of brain

metastasis, modulation of bone homeostasis in case of bone metastasis, activation of resident macrophages in the liver which in turn promoted a fibrotic microenvironment [478]. Among other cancers, the contribution of EVs to pre-metastatic niche formation has been particularly studied in the context of melanoma. A first study from Peinado et al. showed that melanoma derived EVs were able to foster pre-metastatic niche formation in the lung by educating BMDCs [479]. Mechanistically, melanoma EVs transfer the oncogene Met to BMDCs inducing upregulation of Met signaling and promoting BMDC mobilization. In addition, EVs favored vascular leakage that in turn promoted metastasis formation. As mentioned, melanoma cells can enter the lymphatic circulation and create metastasis in lymph nodes. Melanoma derived EVs have indeed been found in lymph nodes [480], [481]. A study from Pucci et al. showed that tumor EVs can disseminate via lymphatic vessels, that they first bind macrophages in lymph nodes, but they can also be taken up by B-cell and dendritic cells, contributing to the formation of a pre-metastatic niche in the lymph nodes [480]. Finally, another study from Hood et al. showed that melanoma-derived exosomes home to sentinel lymph nodes and prepare them for metastasis by induction of several metastatic pathways which affect cell recruitment, ECM deposition and vascular proliferation in lymph nodes [481].

Tumor derived EVs play important roles in multiple steps of cancer progression, including tumor growth, invasiveness and metastasis, angiogenesis, formation of a pre-metastatic niche, immune regulation and response to therapy.



## AIM OF THE THESIS AND OVERVIEW OF THE RESULTS

The main goal of my thesis was to further elucidate the role of the tetraspanin CD63 in the sorting of cargoes to ILVs and in the biogenesis of exosomes. A previous work of the host laboratory had shown the importance of CD63 dependent ILV formation in pigment cells; therefore, such model (MNT-1 pigment melanoma cells) was mainly used during my study. At the time I arrived in the host laboratory, an ongoing study of the team was showing that CD63 dependent pathway is required for the sorting of ApoE to ILVs to form amyloid fibrils required for proper melanogenesis. Interestingly, concomitant publications from another group showed that ApoE could act as a negative regulator of melanoma cell migration and metastatic progression. The primary goal of my thesis was to better understand the role of CD63 in ApoE sorting and to profit from these molecular pathways to investigate the role of ApoE on exosomes in melanoma migration. In this context, my work led to the results presented in the following section, which are organized in five subsections.

**Section 1:** “Apolipoprotein E Regulates Amyloid Formation within Endosomes of Pigment Cells”.

During my post-Master internship in the Raposo’s team and at the beginning of my PhD, I participated to this study, led by Guillaume van Niel and published in 2015, that laid the basis of my PhD thesis. Briefly, by analyzing exosomes released by pigment cells, this study identified ApoE as a main regulator of the early stages of pigmentation. Moreover, we could identify a new pathway of secretion of ApoE through exosomes in pigment cells, which is dependent on the tetraspanin CD63.

**Section 2:** “CD63 regulates the sorting of cholesterol into exosomes”. This study represents the main achievement of my PhD and aimed to further elucidate the molecular mechanisms by which CD63 regulates ILV biogenesis and contribute to endosomal homeostasis. In order to answer this question, I used two model cell types, HeLa cells and MNT-1 cells. Briefly, my results show that CD63 regulates the sorting of cholesterol into ILVs and future exosomes. In pigment cells, such pathway would contribute to the sorting of ApoE to ILVs, a step required for pigmentation. This study is currently being finalized for submission to a peer reviewed journal in the coming months.

**Section 3:** “Preliminary results: the retromer complex regulates retrograde transport of ApoE”. This section contains preliminary results, related to the study in Section 2, illustrating the involvement of the retromer complex in the retrograde transport of ApoE, that in turn would impact its exosomal secretion. Such pathway would also contribute, together with CD63, to the intracellular trafficking of cholesterol. If confirmed, these results will be included in the study of the Section 2.

**Section 4:** “In vitro interaction of melanoma derived extracellular vesicles with collagen”. Considering the respective roles of exosomes and ApoE in melanoma progression, this study aimed to investigate the EV populations released by different melanoma cell lines and how the presence of ApoE on these EVs may influence their functions. In particular, we decided to test the capacity of melanoma derived EVs to interact with collagen, a main component of the extracellular matrix, a process that is very poorly characterized. This study is presented in the form of a “short communication” that will be finalized for submission to a peer reviewed journal.

**Section 5:** “Preliminary results: EV-collagen interaction promotes ECM remodeling and influences melanoma cell migration”. This section contains preliminary results, related to the study in Section 4, aiming to investigate if melanoma derived EVs may promote ECM remodeling and influence melanoma cell migration. This study will be used as a basis for further investigations.

# RESULTS



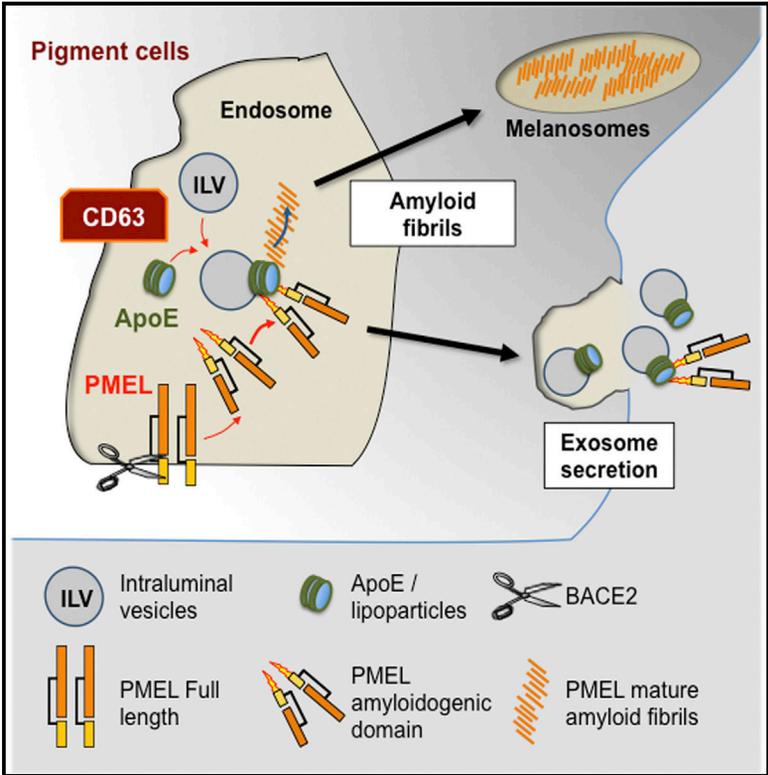
## **Results, Section 1**

Apolipoprotein E Regulates Amyloid Formation within Endosomes of Pigment Cells



## Apolipoprotein E Regulates Amyloid Formation within Endosomes of Pigment Cells

### Graphical Abstract



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### In Brief

Amyloids are protein aggregates that can be cytotoxic. van Niel et al. showed that Apolipoprotein E, a key factor involved in the metabolism of these pathological amyloids, regulates the formation in a non-toxic manner of amyloid structures that serves physiological functions in specialized cells.

### Highlights

- Exosomes and endosomal intraluminal vesicles are associated with ApoE
- ApoE regulates the formation of PMEL amyloid fibrils in endosomes
- ApoE supports the ESCRT independent sorting of PMEL to ILVs

# Apolipoprotein E Regulates Amyloid Formation within Endosomes of Pigment Cells

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## SUMMARY

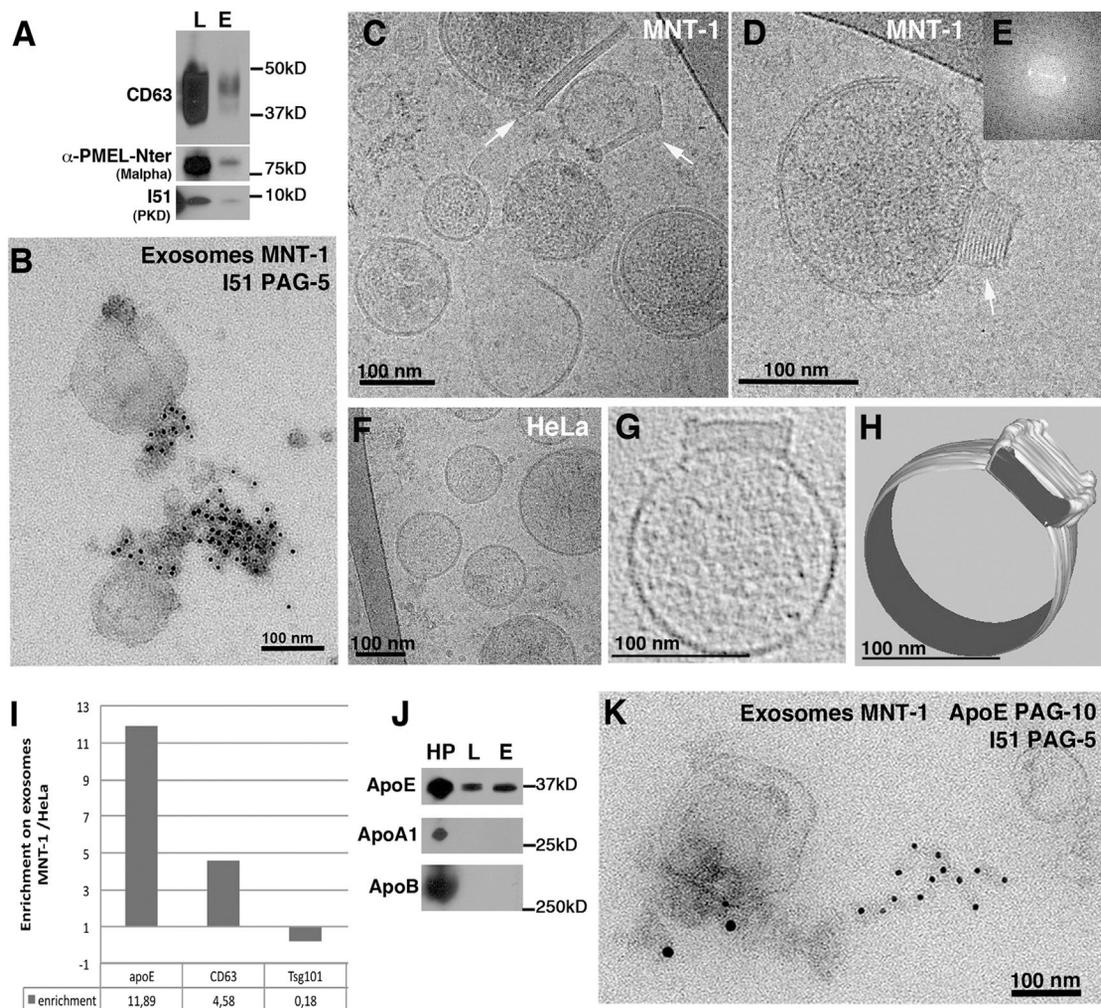
Accumulation of toxic amyloid oligomers is a key feature in the pathogenesis of amyloid-related diseases. Formation of mature amyloid fibrils is one defense mechanism to neutralize toxic prefibrillar oligomers. This mechanism is notably influenced by *apolipoprotein E* variants. Cells that produce mature amyloid fibrils to serve physiological functions must exploit specific mechanisms to avoid potential accumulation of toxic species. Pigment cells have tuned their endosomes to maximize the formation of functional amyloid from the protein PMEL. Here, we show that ApoE is associated with intraluminal vesicles (ILV) within endosomes and remain associated with ILVs when they are secreted as exosomes. ApoE functions in the ESCRT-independent sorting mechanism of PMEL onto ILVs and regulates the endosomal formation of PMEL amyloid fibrils in vitro and in vivo. This process secures the physiological formation of amyloid fibrils by exploiting ILVs as amyloid nucleating platforms.

## INTRODUCTION

Amyloids oligomers and fibrils are broadly associated with various diseases including Alzheimer disease (AD) among others (Harrison et al., 2007). Yet, the process of amyloid formation and the role they play in the physiopathology of disease remains unclear. Of interest, there is accumulating evidence for the involvement of endosomal compartments in amyloid homeostasis (Thinakaran and Koo, 2008). In particular, amyloidogenic proteins and peptides accumulate in multivesicular en-

dosomes (MVEs) and aggregate as toxic oligomers (Takahashi et al., 2002). These endosomal aggregates cause neuronal lysis (D'Andrea et al., 2001) that contributes to the release of toxic species into the extracellular space (Haass and Selkoe, 2007). A cell defense mechanism to neutralize these toxic species is their incorporation into more inert mature amyloid fibrils (Haass and Selkoe, 2007). The fibrillation process is notably influenced by lipoparticles containing the apolipoprotein E (ApoE) (Kane-kiyo et al., 2014), variants of which are major genetic risk factors for AD.

Yet, not all amyloids are associated with disease as so-called "functional" amyloids (Fowler et al., 2007) serve physiological roles. The best studied example of functional amyloids in mammals is amyloid fibrils produced from amyloidogenic fragments of the premelanosomal protein (PMEL) in pigment cells (Watt et al., 2013). These amyloid fibrils serve as a scaffold on which melanins polymerize within lysosome-related organelles called melanosomes (Raposo and Marks, 2007). The unique mechanisms exploited by pigment cells to avoid accumulation of potentially toxic amyloidogenic fragments of PMEL in MVEs remain elusive (Watt et al., 2011, 2013). Yet, the sorting of PMEL amyloidogenic fragments to intraluminal membrane vesicles (ILVs) seem crucial to induce their aggregation into inert "functional" amyloid fibrils (van Niel et al., 2011). During this process, the PMEL luminal amyloidogenic fragment is released by a beta-secretase BACE-2-mediated cleavage (Rochin et al., 2013) from a C-terminal fragment (CTF) (Kummer et al., 2009; van Niel et al., 2011) and, within the same MVE, is sorted to the intraluminal vesicles (ILVs) by an endosomal sorting complex required for transport (ESCRT)-independent/CD63-dependent mechanism (van Niel et al., 2011). Here, we show that ILVs of MVEs function as nucleating platforms for amyloid fibrillogenesis, and ApoE at the membrane of ILVs plays a pivotal role in PMEL sorting and in the formation of PMEL fibrils.



**Figure 1. Identification and Characterization of Exosomes from MNT-1 Cells**

(A) Lysates (L) and exosomes (E) isolated from medium of MNT-1 cells were analyzed by western blot for CD63, PMEL luminal domain full length ( $\alpha$ -PMEL-Nter), and PMEL amyloidogenic fragment (I51).

(B) Exosomes from MNT-1 were immunolabeled with antibodies against PMEL amyloidogenic fragment (I51), revealed with Protein A gold particles (PAG) of 5 nm diameter and observed by EM.

(C–H) Analysis of exosomes by cryo-EM. (C and D) Exosomes purified from MNT-1 cells culture media. Some exosomes are covered by a cap made of a stack of layers (white arrows). (E) Fourier transform of the stack of layers observed in (D) showing diffraction spots at 1/3.5 nm. (F) Exosomes purified from HeLa cells. (G and H) 3D reconstruction of MNT-1 exosomes by cryotomography and corresponding 3D model.

(I) The presence of ApoE, CD63, and Tsg101 in equivalent amount of exosomes from MNT-1 and HeLa cells was determined from western blot analysis (shown in Figure S1H) and their enrichment in MNT-1-derived exosomes was quantified and normalized to their enrichment in exosomes from HeLa cells without cap.

(J) Human Plasma (HP), lysates (L) and exosomes (E) from MNT-1 cells were analyzed by western blot for ApoE, ApoA1, and ApoB.

(K) Exosomes from MNT-1 were co-immunolabeled with antibodies against PMEL amyloidogenic fragment (I51, PAG 15) and ApoE (PAG 10).

See also Figure S1.

## RESULTS

### Exosomes from Pigment Cells Are Associated with ApoE Lipoparticles

To better understand the involvement of ILVs in the formation of amyloids, we first investigated the fraction of ILVs secreted as exosomes from pigment cells. Exosomes are extracellular ILVs that are released after the fusion of MVEs with the plasma membrane (Raposo and Stoorvogel, 2013). They are enriched in specific sets of lipids, proteins, and RNAs and provide details on key

processes in MVEs from which they originate. Exosomes isolated from culture medium of human melanocytic cell line MNT-1 contained the tetraspanin CD63 that is commonly enriched in MVEs and exosomes. The exosome pellet also contained processed and full-length amyloidogenic luminal domain of PMEL (Figure 1A) and the PMEL CTF and the melanosome protein MART-1 (Figure S1A). The distinct intra-endosomal trafficking of PMEL CTF and luminal domain (van Niel et al., 2011) and the absence of interaction between both fragments (Figure S1B) indicated that the PMEL luminal domain is likely

associated with exosomes after release from the CTF. By immunoelectron microscopy (IEM), the processed PMEL amyloidogenic domain associated as aggregates with the outer membrane of 100–150 nm vesicles (Figure 1B). These observations suggested that these exosomes are the extracellular counterpart to the ILVs upon which PMEL amyloid fibrils formed within MVEs. We next analyzed MNT-1 exosomes at high resolution by cryoelectron microscopy (cryo-EM). Exosomes appeared as a population of spherical vesicles of 120 nm diameter on average with few larger vesicles as previously reported (Brouwers et al., 2013; Ji et al., 2013). The vesicles displayed two layers of electronic densities 3.5–4 nm apart, corresponding to each leaflet of the lipid bilayer and were covered by darker densities that correspond to membrane proteins (Figure 1C). Surprisingly, ~10% of MNT-1 exosomes exhibited a “cap” consisting of a stack of horizontal layers (Figures 1C and 1D). Most caps were rectangular by thin section but some were conical (Figures S1D and S1E) and their dimensions range between 12–38 nm in height and 30–100 nm in width (Figures 1C and S1C–S1E). The number of layers varied from 4 to 12, but the distance between each layer was constant to 3.5 nm, as shown by the diffraction spots present in the Fourier transform (Figure 1E, inset). Analysis of the exosomes by cryotomography revealed that the caps had a closed cylindrical shape in three dimensions (Figures 1G and 1H). Such caps were also observed on exosomes from primary melanocyte cells but not on exosomes from HeLa (Figure 1F) and other cell types (Figure S1F).

The stacked layers and spacing of the MNT-1 exosome caps were strikingly reminiscent of low-density lipoproteins consisting of apolipoproteins and cholesterol forming a striated core with layers spaced by 30–35 Å and observed by cryo-EM (Orlova et al., 1999). Using quantitative mass spectrometry (SILAC) we compared exosomes derived from either unlabeled MNT-1 cells or HeLa cells cultured in heavy media. In the genome ontology (GO) term 0034358 “Plasma lipoprotein particle,” only human ApoE was found statistically enriched in exosomes derived from MNT-1 (enrichment factor: 4.30,  $p$  value =  $1.08\text{e-}03$ , 25 specific peptides with a coverage >61%) in agreement with endogenous expression of APOE in pigment cell type (Ishida et al., 2004).

Western blot analysis of exosomes released from MNT-1 cells further shows that ApoE was enriched (Figures 1I and S1G) and that apolipoproteins A1 and B were absent (Figure 1J), excluding potential contamination by plasma lipoparticles. Moreover, floatation of exosomes on sucrose gradient showed that ApoE was only present in the fraction containing exosome-associated proteins, thereby excluding free ApoE or ApoE-lipoparticles in the exosomal pellet (Figure S1H). Using cryo-EM and immunogold labeling we strengthen the findings that ApoE is associated with exosomes (Figure 1L). Immunogold labeling on aldehyde-fixed exosomes highlights the co-localization of ApoE on the outer membrane of exosomes with CD63 (Figure S1I) and PMEL fibrils (Figure 1K). Similar observations were done on exosomes derived from human primary melanocytes (Figures S1J and S1K).

### Endogenous ApoE Co-localizes and Interacts with PMEL on ILVs of Endosomes

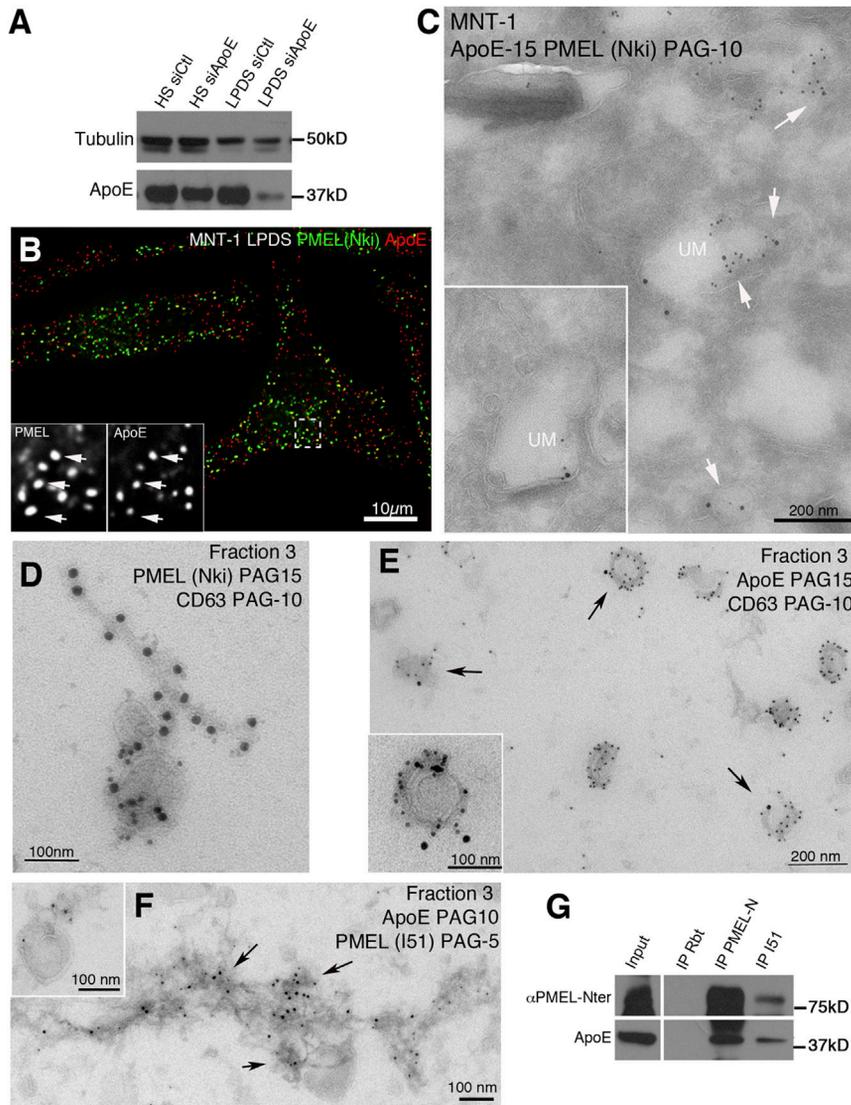
The endosomal origin of exosomes led us to investigate whether ApoE is present within MVEs. Endogenous ApoE was detected

by western blotting of lysates of MNT-1 cells grown in lipoprotein-depleted human serum (LPDS) and was significantly reduced after treatment with ApoE-specific small interfering RNA (siRNA) (Figure 2A). By immunofluorescence microscopy, labeling for PMEL and ApoE partially overlapped in post-Golgi compartments of MNT-1 cells grown in LPDS (Figure 2B), and IEM analyses detected ApoE in MVEs and in early stage melanosomes containing PMEL-derived fibrils (Figure 2C). The association of ApoE with ILVs was confirmed by cell fractionation on sucrose gradients, in which CD63, PMEL amyloidogenic fragments, and ApoE were enriched in a fraction of similar density to that of exosomes (Figure S2A, fraction 3). IEM analysis of this fraction revealed membrane vesicles enriched in CD63 (Figures 2D and 2E) that were of similar diameter and orientation as the exosomes described above. These ILVs associated with PMEL fibrils (Figures 2D and S2B) and ApoE-positive structures (Figures 2E and 2F). The co-localization of ApoE and PMEL on ILVs was consistent with their co-immunoprecipitation using antibodies against the luminal domain and amyloidogenic region of PMEL (Figure 2G). These data indicate that ApoE is associated with the PMEL amyloid luminal domain and PMEL fibrils at the surface of ILVs within MVEs.

### ApoE Regulates PMEL Amyloid Fibrils Formation

The reported role of ApoE in pathological amyloidogenesis (Kaneke et al., 2014) prompted us to investigate the capacity of ApoE to regulate PMEL fibrillogenesis within MVEs of MNT-1 cells. Depletion of endogenous ApoE by siRNA, but not of exogenous ApoE using LPDS (Figure 3A), strongly reduced both the formation of epitopes associated with amyloid fibrils (Figure 3A) and the accumulation of PMEL fibrils in unpigmented stage II melanosomes observed by EM (Figures 3B and 3C). Instead of forming structured fibrils, the PMEL luminal domain in ApoE siRNA-treated cells accumulated as unstructured aggregates within vacuolar structures (Figures S3A and S3B). Consequently, relative to control cells the number of stage III and IV melanosomes with melanin pigments deposited along the fibrils (Seiji et al., 1963) was reduced and aberrant multilamellar structures with irregular luminal pigment deposits appeared (Figures 3B and 3C, noted “APM,” and S3A). Despite the altered morphology of melanosomes, ApoE siRNA did not affect total melanin content (Figure S3C), suggesting that the effect was uniquely associated to amyloid fibril formation and not melanin synthesis. Similar impairment of generation of PMEL fibrils within early melanosomes was also observed after depletion of endogenous ApoE in human primary melanocytes (Figures 3D and 3E).

To test whether ApoE depletion affects PMEL amyloidogenesis in a physiological context, we analyzed melanosome morphology in pigment cells in *APOE*<sup>-/-</sup> mice. Compared to control C57BL/6J mice (wild-type [WT]), *APOE*<sup>-/-</sup> mice did not display any obvious coat color defect (Figure S4A) as reported in *PMEL*<sup>-/-</sup> mice (Hellström et al., 2011). Because of potential compensatory effects observed in skin melanocytes of mice with melanogenesis defects (Lopes et al., 2007), we focused on retinal pigment epithelium (RPE) in which the compensatory effect is less apparent. Whereas, melanosomes in the RPE of control mice were characteristically ellipsoidal, melanosomes of *APOE*<sup>-/-</sup> mice were rounder (Figures 4A and 4C), but neither larger nor fewer in



**Figure 2. Endosomal Localization of ApoE**

(A) Whole cell lysates of MNT-1 cells that were cultured in the presence of human serum (HS) or lipoprotein-depleted human serum (LPDS) and treated with control siRNA or ApoE siRNA were analyzed by western blot using an anti-ApoE antibody or anti- $\beta$ -tubulin antibody as a loading control.

(B) MNT-1 cells cultured in LPDS were analyzed by IFM after labeling for PMEL luminal domain (Nki) and ApoE; arrows point to co-localization spots. (C) Ultrathin cryosections of MNT-1 cells were double immunogold labeled for PMEL luminal domain (Nki, PAG 10) and ApoE (PAG 15). Arrows indicate ApoE labeling in unpigmented early melanosomes (UM).

(D–F) Fraction 3 obtained from cell fractionation of MNT-1 cells on sucrose gradients (Figure S2A) was observed by electron microscopy after immunogold labeling with antibodies against (D) PMEL luminal domain (Nki, PAG 15) and CD63 (PAG 10), (E, inset) ApoE (PAG 15) and CD63 (PAG 10), or (E, inset) ApoE (PAG 15) and PMEL amyloidogenic fragment (I51 PAG 5).

(G) TX-100 lysates of MNT-1 cells were immunoprecipitated with isotype control antibody or antibodies specific to PMEL luminal domain full length ( $\alpha$ -PMEL-Nter) and PMEL amyloidogenic fragment (I51) as indicated. Immunoprecipitates (IP) or untreated lysate (Input) were analyzed by western blot with  $\alpha$ -PMEL-N (top) or anti-ApoE antibody (bottom).

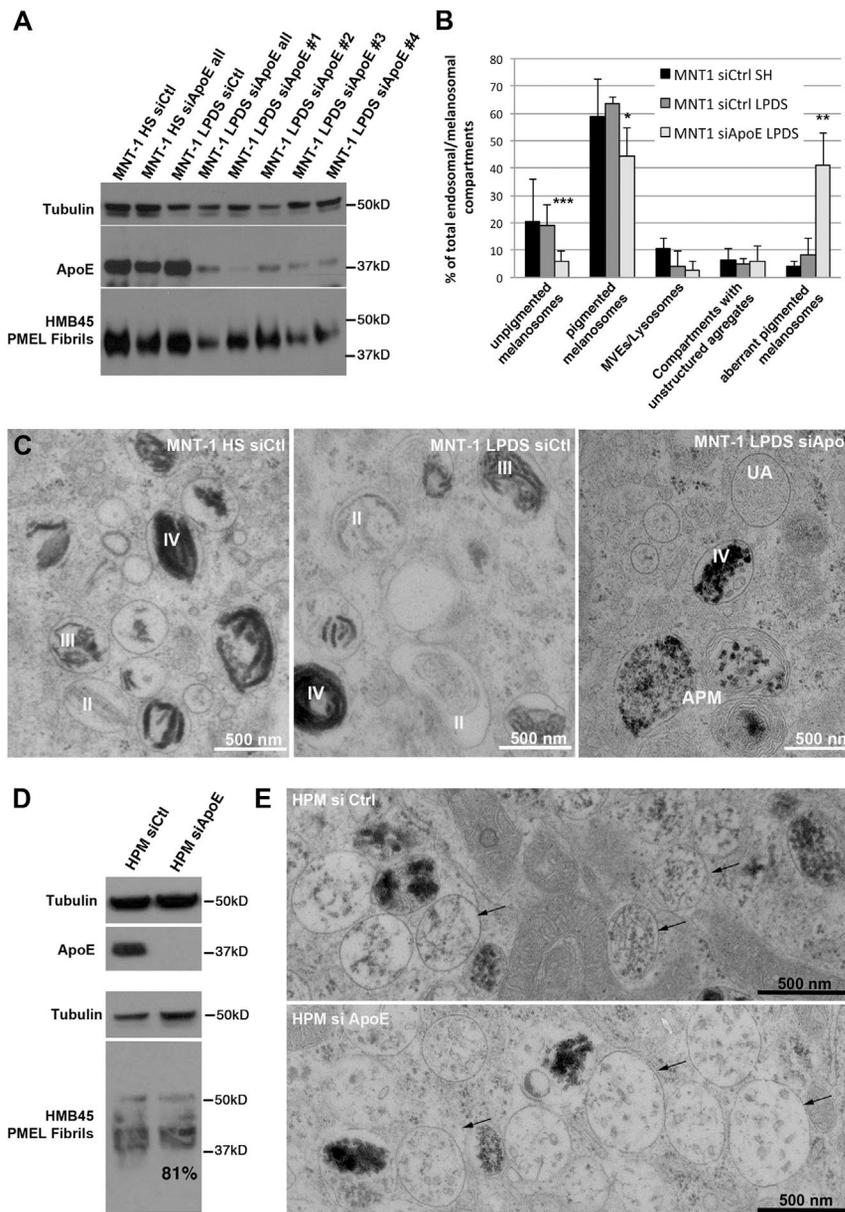
See also Figure S2.

number (Figures S4B and S4C). The round shape is characteristic of a defect in PMEL-derived amyloidogenesis as observed in *PMEL*<sup>-/-</sup> mice (Hellström et al., 2011). Importantly, overexpression of human *APOE3* isoform, and to a lesser extent of human *APOE4* isoform, rescued the phenotype of *APOE*<sup>-/-</sup> mice (Figures 4B and 4C). In vivo, ApoE- and PMEL-positive labeling were also observed in MVEs of RPE likely corresponding to early stage melanosomes (see electron micrographs in Figures 4D, 4E, and S4D) and on extracellular vesicles that were by their size reminiscent of exosomes in the vicinity of RPE cells (Figures 4F and S4E). These data indicate that ApoE is associated with ILVs and exosomes and is required for PMEL amyloidogenesis and consequently melanosome maturation in vivo.

#### ApoE Is Involved in the CD63-Dependent/ESCRT-Independent Sorting of PMEL

The defects in melanosome morphology observed in ApoE-deficient melanocytes are reminiscent of those observed when

linked to PMEL sorting to ILVs since siRNA-mediated depletion of ApoE reduced the association of ApoE and PMEL luminal domain to ILVs that are released as exosomes (Figure 5A). This defect of PMEL sorting in ApoE-depleted cells echoed the observations made upon depletion of CD63, which regulates the ESCRT-I-independent sorting of the PMEL luminal domain on ILVs (van Niel et al., 2011). In agreement with a role of ApoE and CD63 in the same sorting pathway, partial siRNA-mediated depletion of CD63 (Figure S5B) induced a significant accumulation of ApoE in a perinuclear compartment (Figure 5B) that we identified by EM as the Golgi apparatus (Figure 5C). In contrast, depletion of the ESCRT-I subunit TSG101 (Figure S5B) induced an accumulation of ApoE and PMEL in the lumen of enlarged endosomes with irregular internal membranes (Figures S5C and S5D). As a potential consequence of impaired endosomal trafficking of ApoE, depletion of CD63, but not of ESCRT-I, inhibited the targeting of ApoE to ILVs secreted as exosomes (Figure 5D) and the co-immunoprecipitation of PMEL with ApoE



**Figure 3. ApoE in the Fibrillogenesis of PMEL in Cell Line**

(A) Whole cell lysates of MNT-1 cells that were cultured in HS or LPDS and treated with either control siRNA or different ApoE siRNA were analyzed by western blot with anti-ApoE antibody or anti- $\beta$ -tubulin antibody as a loading control. Corresponding Triton X-100-insoluble fraction from MNT-1 cells were analyzed by western blot using fibrils-specific antibody HMB45.

(B) Quantification of the number of different endosomal/melanosomal compartments, as indicated on the x axis and defined by morphology, observed by conventional EM in MNT-1 cells that were treated as indicated. Values represent the percentage of each compartment ( $\pm$ SD) relative to all of the endosomal/melanosomal compartments identified in 20 cell profiles per condition (\*\*\* $p < 0.01$ , \*\* $p < 0.02$ , \* $p < 0.05$ ).

(C) Control cells cultured in HS and LPDS or in ApoE-depleted cells cultured in LPDS observed by conventional EM. Labels II, III, and IV, indicate the different stages of maturation of melanosomes; UA, unstructured aggregates; APM, aberrant pigmented melanosomes.

(D) Whole cell lysates of human primary melanocytes that were treated with either control siRNA or ApoE siRNA were analyzed by western blot with anti-ApoE antibody or anti- $\beta$ -tubulin antibody as a loading control. Corresponding Triton X-100 insoluble fractions were analyzed by western blot using fibrils-specific antibody HMB45.

(E) Control and ApoE-depleted human primary melanocytes were observed by conventional EM. Arrows indicate unpigmented early melanosomes that are devoid of PMEL fibrils after ApoE depletion. See also Figure S3.

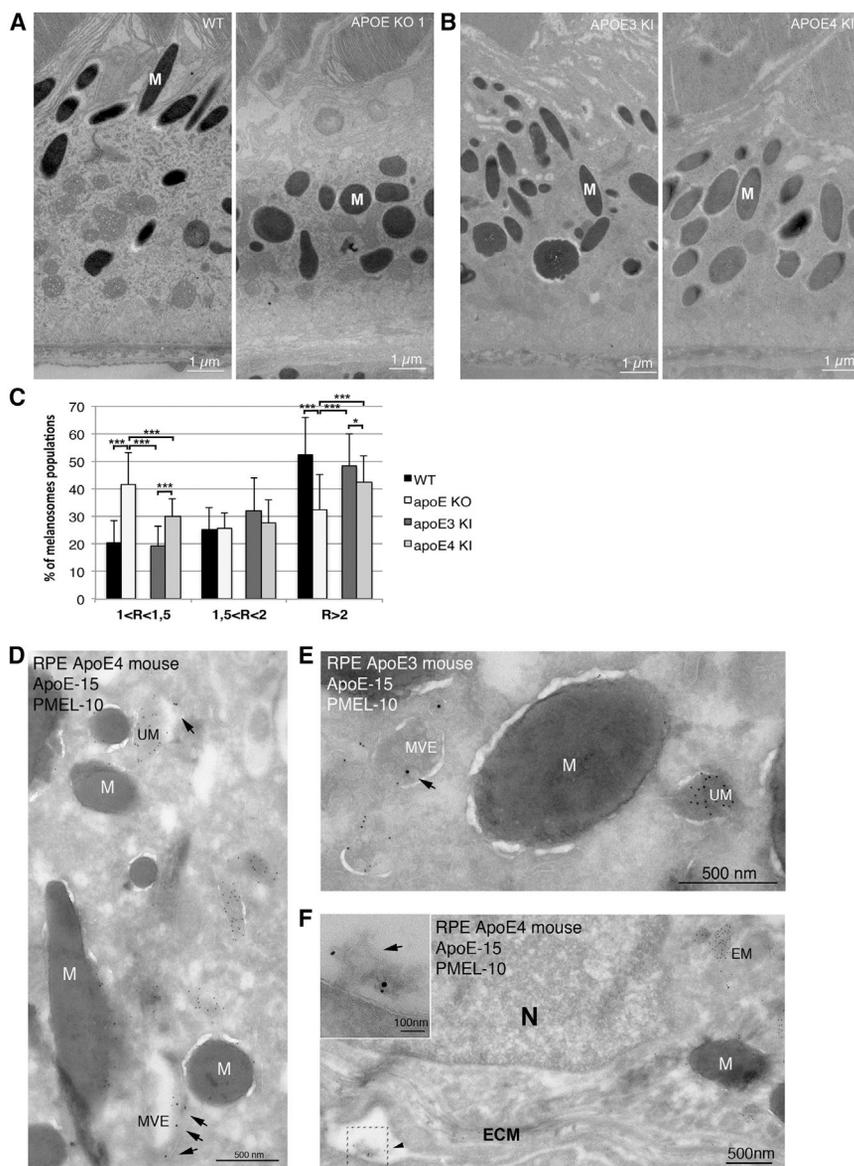
(Figure S5E). By contrast, inhibition of sphingomyelinase—that inhibits ESCRT-independent ILV formation in glial cells (Trajkovic et al., 2008) but not PMEL sorting (van Niel et al., 2011)—did not impair ApoE association with exosomes (Figure S5F), indicating that ApoE sorting to ILVs is ceramide-independent. Our results show that ApoE is a downstream effector of the ESCRT-independent/CD63-dependent sorting of PMEL that regulates PMEL amyloidogenesis.

## DISCUSSION

Here, we show that the binding of ApoE to the membrane of exosomes and ILVs is a major regulator of PMEL amyloid fibrils formation during melanogenesis. This ascertains APOE as a new

pigmentation gene, in agreement with the regulation of its expression with MITF, a master regulator of melanocyte development (Hoek et al., 2008). As with other genes required for early steps of melanogenesis (Hellström et al., 2011), APOE might have been missed because its disruption causes only minor coat color alterations and no visual defect (Mak et al., 2014) due to potential compensatory mechanisms.

ApoE is an amphipathic protein (Segrest et al., 1992) that could interact both with membranes of ILVs and with the PMEL luminal domain during the processes of sorting and fibrillation. This suggests that ApoE could act as a loading device on ILVs for shed PMEL luminal domain providing a mechanism for the CD63-dependent/ESCRT-independent sorting pathway of PMEL (van Niel et al., 2011). The involvement of CD63 in ESCRT-independent ILVs formation in a cervical cancer cell line (Edgar et al., 2014) and the potential presence of ApoE in exosomes secreted by other cell types such as oligodendrocytes (Conde-Vancells et al., 2008; Krämer-Albers et al., 2007) suggests a widespread mechanism where ApoE could act as a



**Figure 4. ApoE in the Fibrillogenesis of PMEL In Vivo**

(A) RPE sections from *APOE*<sup>+/+</sup> (left) and *APOE*<sup>-/-</sup> mice (right) were analyzed by conventional EM. Note the ellipsoidal melanosomes (M) in RPE of *APOE*<sup>+/+</sup> mice and their rounder shapes in the RPE of *APOE*<sup>-/-</sup> mice.

(B) RPE sections from *APOE3* (left) and *APOE4* Knockin mice (right) were analyzed by conventional EM. Note the ellipsoidal melanosomes (M) in both RPE.

(C) The ratio between maximum width and length of an average of 300 melanosomes per conditions was measured using ITEM software.

(D-F) Ultrathin cryosections of RPE from *APOE3* and *APOE4* KI mice were double immunogold-labeled for PMEL luminal domain (HMB45, PAG 10) and ApoE (PAG 15). (D and E) Arrows indicate ApoE labeling in MVE and unpigmented early melanosomes (UM). (F) Arrows indicate extracellular vesicles labeled for PMEL and ApoE in the vicinity of RPE (\*\*p < 0.01, \*\*p < 0.02, \*p < 0.05). See also Figure S4.

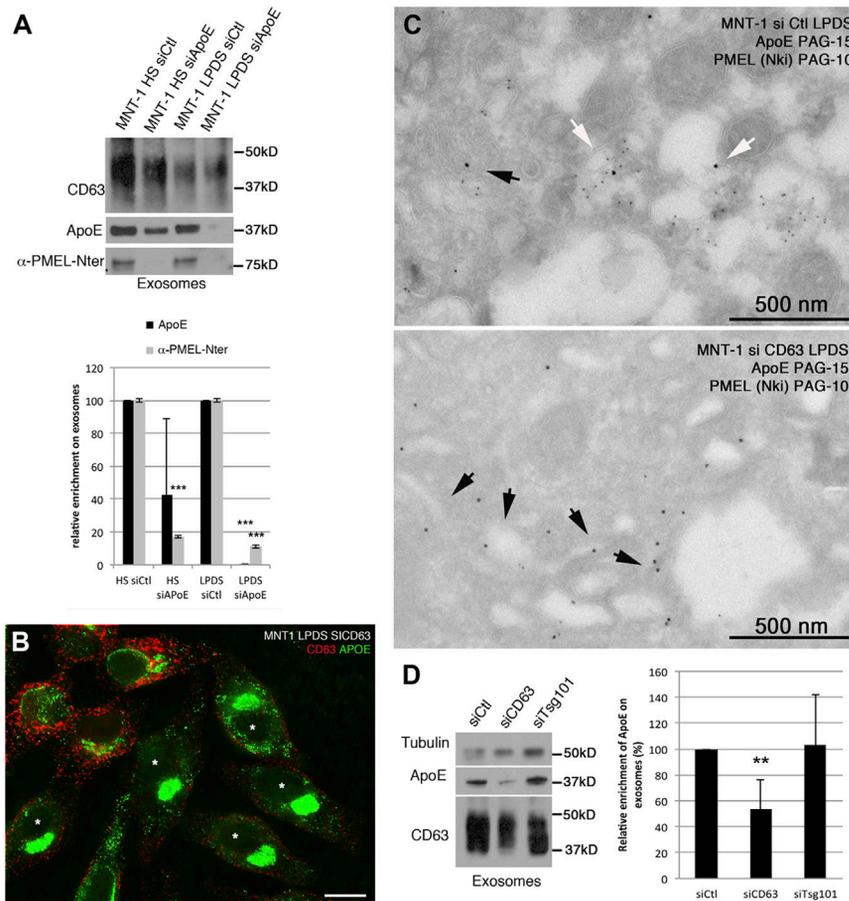
fibrils. By acting directly at the site of production of PMEL-derived amyloid peptides, ApoE would prevent the accumulation of potentially toxic PMEL-derived amyloid peptides. Interestingly, the neural crest origin of melanocytes and the analogies of trafficking and processing pathways between PMEL and APP strengthen the relevance of this process for amyloid formation in AD. In this context, however, the major expression of ApoE and Aβ by distinct cell types, astrocytes and neurons, respectively (Xu et al., 2006), prevents similar process as ApoE and Aβ would mainly encounter in the extracellular medium. It is then tempting to speculate, as observed separately for exosomes (Yuyama et al., 2014) and ApoE (Li et al., 2012), that exosomes

general loading mechanism for soluble proteins or shed luminal domains of endosomal transmembrane proteins. ApoE could also create specific regions on the membrane of ILVs and exosomes, as shown by cryo-EM that would cluster important co-factors of fibrillation. Among them, specific lipids, such as ganglioside or cholesterol, have already been reported as regulators of amyloidogenesis processes at the surface of exosomes (Yuyama et al., 2014). In view of the differential level of rescue of PMEL amyloidogenesis by *APOE3* and *APOE4* expression in vivo, the investigation of the influence of *APOE* isoforms on such processes would be of interest.

Moreover, our data suggest that the presence of ApoE in endosomes is one of the specific mechanisms that pigment cells use to avoid potential toxicity inherent to PMEL amyloidogenesis. Within MVEs, ApoE enables ILVs to nucleate newly shed PMEL prefibrillar species (Watt et al., 2011) into mature amyloid

harboring ApoE would favor endocytosis and targeting of Aβ to MVEs and provide a favorable environment for Aβ seeding and neutralization (Hu et al., 2009).

The association of ApoE with ILVs and exosomes ascertains a new pathway of secretion for ApoE that would differ from its typical release as phospholipid discs (Kockx et al., 2008). The association of ApoE with ILVs would also extend the properties of exosomes as ApoE binds specific receptors for uptake and modulates the activity of soluble ApoE receptors (Rebeck et al., 2006). This association also prompts us to reconsider the respective roles of ApoE and exosomes in several pigment cell-associated pathologies such as age-related macular degeneration (Johnson et al., 2011; Wang et al., 2009) and melanoma metastasis (Peinado et al., 2012; Pencheva et al., 2012) where ApoE may endow signaling properties to exosomes.



**Figure 5. ApoE in CD63-Dependent Sorting of PMEL**

(A) Top: exosomes from MNT-1 cells that were cultured in HS or LPDS and treated with control siRNA or ApoE siRNA were analyzed by western blot with anti-CD63, anti-ApoE, and anti-PMEL luminal domain ( $\alpha$ -PMEL-Nter antibody). Bottom: relative enrichment of ApoE and PMEL luminal domain was quantified and results are expressed as the mean  $\pm$  SD of three experiments (\*\* $p < 0.01$ ).

(B) MNT-1 cells in LPDS and treated with CD63 siRNA were analyzed by IFM after labeling for CD63 and ApoE. White stars indicate CD63-inactivated cells; we purposely show an experiment in which CD63 depletion was lower than normal to emphasize the differences between cells that do and do not express CD63. Scale bar, 10  $\mu$ m.

(C) Ultrathin cryosections of MNT-1 cells that were cultured in LPDS and treated with control siRNA (top) or CD63 siRNA (bottom) were double immunogold labeled for PMEL luminal domain (Nki, PAG 10) and ApoE (PAG 15). White arrows indicate ApoE labeling in unpigmented melanosomes and black arrows indicate ApoE-labeling in Golgi stack.

(D) Exosomes secreted by MNT-1 cells that were cultured in LPDS and treated with control siRNA, CD63 siRNA, or tsg101 siRNA were analyzed by western blot with anti-CD63, anti-ApoE, and or anti- $\beta$ -tubulin antibody as a loading control. Right: relative enrichment of ApoE was quantified and results are expressed as the mean  $\pm$  SD of three experiments (\*\* $p < 0.02$ ). See also Figure S5.

## EXPERIMENTAL PROCEDURES

### Mice

Male *APOE*<sup>-/-</sup> mice (n = 3) and male C57/bl6/J (n = 3) were purchased from the Maastricht University. Mice were 12 weeks of age when sacrificed. Until sacrifice, mice were housed in cages with free access to water and regular chow diet (AB diets, Woerden). *APOE3* and *APOE4* targeted replacement mice (n = 2 each) were purchased from Taconic Europe A/S Lille Skensved. Mice were 12 weeks of age when sacrificed. Until sacrifice, mice were housed in cages with free access to water and regular chow diet (irradiated A4 from Safe-Diet). Before collecting all required tissues, mice were perfused with a sterile saline solution (150 mM saline, 2.5 mM CaCl<sub>2</sub> in HEPES, pH 7.3) via the left ventricle. The eyes (enucleated and extraocular muscles were cut) were dissected and harvested in modified Karnovsky's fixative (2% paraformaldehyde/2% glutaraldehyde/0.1 M cacodylate buffer, pH 7.3/0.06% CaCl<sub>2</sub>). After a few hours, eyes and skin were sectioned and fixed overnight at 4°C in modified Karnovsky's fixative. The experimental animal committee of the Maastricht University approved this animal experiment.

### Cell Culture, Drug Treatment, Transfection, and siRNA Depletion

Human melanocytic MNT1 cells were maintained as previously described (van Niel et al., 2011). When indicated, fetal bovine serum was replaced by human serum and lipoprotein-depleted human serum (Millipore). Cells were treated for 24 hr with 2.5  $\mu$ M GW4869 (Sigma). MNT1 cells were transfected with siRNA duplex oligonucleotides as reported previously (van Niel et al., 2011). Cells were subjected to one round of siRNA transfection and collected after 72 hr. MNT-1 cells were transfected with plasmid constructs using Lipofectamine 2000 (Invitrogen) following the manufacturer's recommendations and cells were collected and analyzed after 48 hr. For the rescue experiment, cells

were subjected to two rounds of siRNA transfection against ApoE and after 72 hr cells were transfected with plasmid constructs for ApoE3 and collected after 24 hr.

### Exosome Isolation

Exosomes were prepared from conditioned media incubated for 48 hr on sub-confluent cells. Media were centrifuged at 2,000  $\times$  g for 15 min (4°C) and 4,000  $\times$  g (15 min, 4°C) to remove debris. Next, the supernatant was centrifuged at 10,000  $\times$  g for 30 min (4°C) and exosomes were collected from the supernatant by centrifugation at 100,000  $\times$  g for 60 min (4°C). The pellet was resuspended and washed in PBS (pH 7).

### Immunoprecipitation

MNT1 cells were lysed directly in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA [pH 7.2]) with protease inhibitor cocktail (Roche). After 30 min at 4°C, insoluble material was removed by centrifugation at 12,000  $\times$  g and cell lysate was precleared for 30 min by addition of 50  $\mu$ l protein G-Agarose beads (Invitrogen) and for 30 min by addition of 50  $\mu$ l protein G-Agarose beads coated with irrelevant rabbit antibody (Dako). Proteins were then immunoprecipitated by adding 50  $\mu$ l protein G-Agarose beads coated with antibodies in MNT-1 lysates and by adding 50  $\mu$ l protein G-Agarose beads coated with specific antibody for anti-ApoE antibody in HeLa cell lysates. After 2 hr at 4°C under constant agitation, beads were washed five times in lysis buffer. The immunoprecipitates were subjected to western blot analysis as described below.

### Western Blot

Triton X-100 soluble and insoluble material from MNT-1 cells was obtained as previously described (van Niel et al., 2011) and analyzed by western blot as

described in van Niel et al. (2011). Signal intensities were quantified with ImageJ software.

### Electron Microscopy

Tissues from *APOE*<sup>-/-</sup> mice and wild-type mice and MNT-1 cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for conventional EM analysis. Tissue and cells were processed for Epon embedding and ultrathin sections and then contrasted with uranyl acetate and lead citrate as described (Lopes et al., 2007). For ultrathin cryosectioning and immunogold labeling, samples were processed and analyzed as reported previously (van Niel et al., 2011).

### Cryoelectron Microscopy

A glow-discharged lacey Formvar copper grid (Ted Pella) was loaded with 5  $\mu$ l of exosome solution. Next, the solution was blotted and vitrified in liquid ethane by plunge freezing. Images were recorded under low-dose conditions with a FEI CM120 operating at 120 kV with a SSD GATAN 1 k  $\times$  1 k camera at a magnification of 35,000 resulting in 4.96  $\text{\AA}$ /pixel. The intended underfocus was set to  $-1.5$  to  $-2$  mm. Cryotomograms were recorded using TEMography (JEOL, USA) at 200 kV with a JEOL JEM 2200FS equipped with a  $\Omega$  filter. A 10 eV energy window was used to record Z-loss filtered images. Cryotomograms were collected at 1.5 $^\circ$  increments over 100 $^\circ$  range at 25,000 $\times$  magnification. Images were recorded at  $-5$  to  $-7$   $\mu$ m underfocus, binning 1, corresponding to 4  $\text{\AA}$ /pixel using an Ultrascan 100 GATAN CCD camera. The total dose was set to 80 electrons/ $\text{\AA}^2$ . Tilt series alignment and weighted back-projection reconstruction were performed with the aid of the gold beads using *etomo* (IMOD) software. Reconstructed structures were computationally identified with the isosurface selection tool in IMOD.

### Image Analysis and Quantification

Melanosome stages were defined by morphology (van Niel et al., 2011). Quantification of the length and width of melanosomes was determined using the ITEM software (Soft Imaging System [SIS]).

Antibodies list, siRNA list, exosome isolation on optiprep gradient, cell fractionation, melanin assay, genotyping, immunofluorescence microscopy, proteomic analysis, cell culture and SILAC labeling (metabolic labeling and exosome purification), in-gel digestion, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis, and processing of MS data are described in the Supplemental Experimental Procedures.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2015.08.057>.

### AUTHOR CONTRIBUTIONS

G.v.N. conceived, designed, performed, and analyzed data from all experiments and wrote and edited the manuscript. P.B. performed and analyzed conventional electron microscopy. A.d.C. and C.F. performed cryoelectron microscopy. A.d.C. and I.H. performed cryotomography and 3D reconstruction. A.L.C. performed isolation and biochemical analysis of exosomes. R.P. performed analysis on primary melanocytes. L.S. and M.C.P. provided biopsies from mice. F.D. carried out the MS experimental work. L.D. supervised MS and proteomic data analysis. D.L. and G.R. analyzed data, wrote, and edited the manuscript. D.L. and G.R. contributed equally to this work.

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## Results, Section 2

CD63 regulates the sorting of cholesterol into exosomes



## CD63 REGULATES THE SORTING OF CHOLESTEROL INTO EXOSOMES

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### Abstract

Exosomes are extracellular vesicles that function as a clearance cellular pathway and in intercellular communication. The function of exosomes is tightly related to their composition, resulting from the active sorting of cargoes during their biogenesis as intraluminal vesicles (ILVs) in multivesicular endosomes (MVEs). While the mechanisms supporting protein sorting in exosomes are well characterized, the sorting of lipids has been overlooked. Yet, cholesterol enrichment on exosomes is a shared feature that likely contributes to their functions and that would modulate cellular sterol homeostasis. We show here that the tetraspanin CD63 is a key regulator of cholesterol sorting on exosomes during their biogenesis. Absence of CD63 prevents targeting of cholesterol to intraluminal vesicles and stimulates its retrieval from MVEs and its targeting to the Trans-Golgi Network by actin mediated vesicular transport. Our work illustrates a balance between inward sorting and retrieval at MVEs that regulates cholesterol homeostasis, impacts exosomes composition and has a physiological relevance in processes such as pigmentation.

## Introduction

Exosomes are small extracellular vesicles (EVs) released by most cell types and playing key roles in cell-cell communication. The fates and functions of exosomes rely on their specific composition that results from the action of sorting mechanisms during their generation. Exosomes are generated as intraluminal vesicles (ILVs) within multivesicular endosomes (MVEs) (Palmulli and van Niel, 2018). Such process requires the enrichment of cargoes, fated for exosomal secretion, in specific microdomains on the delimiting membrane of MVEs and the subsequent inward budding of these microdomains. These two steps are mediated by different mechanisms which can be ESCRT-dependent or -independent (Baietti et al., 2012; Colombo et al., 2013; van Niel et al., 2011). The exosome composition varies according to the producing cell type but common features such as the enrichment of members of the tetraspanin family (Escola et al., 1998) and cholesterol (Möbius et al., 2003) are shared among most exosome populations studied so far. While the mechanisms supporting protein sorting to exosomes are pretty well understood, the ones driving enrichment of lipids such as cholesterol are not known.

Cholesterol contributes to membrane organization and fluidity and regulates membrane trafficking and signaling processes (Ikonen, 2008). Cholesterol trafficking and metabolism is tightly regulated in order to maintain an uneven distribution between intracellular compartments, with the majority of cholesterol being present at the plasma membrane (Iaea and Maxfield, 2015) and intermediate concentrations being in recycling endosomes and in ILVs of MVEs (Möbius et al., 2003). In endosomes, cholesterol trafficking has been mainly investigated during its extraction from endocytosed cholesterol-rich low density lipoproteins (LDL), that provide a main source of cholesterol in addition to its de novo biosynthesis (Ikonen, 2008). The export of endosomal cholesterol is regulated by the cooperative action of Niemann Pick Type C1 and 2 proteins (NPC1 and NPC2), that results in the redistribution of cholesterol from LDL to the delimiting membrane of late endosomes. Egress of cholesterol from endosomes employs different mechanisms: either non-vesicular transport, which requires the formation of membrane contact sites (MCS) between different organelles, or vesicular transport, which requires incorporation of cholesterol into transport intermediates (Iaea and Maxfield, 2015). The latter process implies cargo recognition by different machineries, including the retromer complex and sorting nexin proteins, the formation of a bud, the extension of this bud to form a tubule or a vesicle and their final scission to release the transport carrier, these last steps requiring actin polymerization (Simonetti and Cullen, 2019). While these retrieval pathways of cholesterol from endosomes may impact ILV composition, it does not explain how cholesterol is sorted into ILVs and exosomes.

Among the known sorting mechanisms acting at MVEs, the tetraspanin CD63 have been implicated in the ESCRT-independent sorting of specific cargoes such as the melanosomal proteins PMEL (van Niel et al., 2011) and ApoE (van Niel et al., 2015) or the Epstein–Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) (Hurwitz et al., 2017; Verweij et al., 2011). CD63 is a well-known resident protein of late endosomes, lysosomes and lysosome-

related organelles (LROs) that has been involved in the transport of cargoes to the endolysosomal system (Pols and Klumperman, 2009). Nevertheless, the precise role of CD63 in MVEs is still not well understood. As all tetraspanins, CD63 is enriched in specific membrane domains, called tetraspanin enriched microdomains, which integrate a network of partner proteins to regulate their trafficking, cleavage and functions. Importantly, the organization of such microdomains relies on the presence of specific lipids such as gangliosides and cholesterol (Charrin et al., 2003; Odintsova et al., 2006). The association of tetraspanins with cholesterol is further reinforced by the recent description of a cholesterol intramembrane cavity within the transmembrane region of another tetraspanin, CD81, (Zimmerman et al., 2016).

These evidences led us to investigate the role of CD63 in the sorting of cholesterol to ILVs and exosomes. We show here that CD63 contributes to the maintenance of endosomal homeostasis and morphology by regulating the balance between cholesterol sorting into ILVs and cholesterol retrieval from MVE to Golgi Apparatus by outward budding. Such process has a physiological relevance in pigment melanoma cells where it contributes to the sorting of endogenous ApoE to ILVs, hence participating in the early stages of pigmentation and regulating both lipid and protein composition of exosomes.

## Results

### **CD63 depletion do not affect global ILV formation and exosome secretion**

In order to investigate the role of CD63 in exosome biogenesis we first generated CD63 KO HeLa cells using CRISPR/CAS9 technique and we isolated two different clones, hereafter indicated as KO#1 and KO#2 (Fig. S1A). Extracellular vesicles (EVs) from the same number of HeLa WT or CD63 KO cells were isolated by differential ultracentrifugation and the 100,000 g pellet was recovered and analyzed. WB analysis showed a trend for higher levels of exosomal markers such as CD9, alix and syntenin in EVs from CD63 KO cells, which was only significant for Alix in KO#1 (Fig.1A and B). Similarly, cellular content of CD9 and syntenin was not significantly changed in CD63 KO cells, while alix levels were slightly decreased (Fig. S1A and S1B). NTA analysis showed that WT and CD63 KO cells release similar number of exosomes (Fig. S1C) with similar size distribution (Fig. 1C). CD63 has been involved in the biogenesis of small ILVs (less than 40nm) in HeLa cells depleted for the ESCRT-0 component HRS (Edgar et al., 2014). Due to the resolution limits of the NTA, we could not detect EVs smaller than 50nm by this technique, but EM analysis showed only EVs with average size of 100nm-150nm in both conditions (Fig. 2A and S2A). Moreover, 100,000 g pellets were loaded onto a bottom-up density gradient (Fig S1D and S1E). WB analysis of the fractions showed floatation of exosomes at similar densities (between 1.08 g/ml and 1.12 g/ml) for both WT and CD63 KO, suggesting that these cell lines release a homogeneous population of EVs. In line with no obvious defect of exosomes production, WT and CD63 KO HeLa cells presented similar patterns of lamp1 positive compartments (Fig. S1F) and MVEs with similar size and containing similar number of ILVs (Fig. 1D). All together, these data suggested that the absence of CD63 neither induces striking changes of the endolysosomal pathway nor affects global ILV formation and exosome secretion.

### **CD63 regulates the sorting of cholesterol to exosomes**

As CD63 did not seem to participate in exosome generation and secretion per se, we hypothesized that the role of CD63 in MVE generation could be related to its capacity, in pigment cells, to regulate the loading on exosomes of ApoE (van Niel et al., 2015), which is a known regulator of cellular cholesterol homeostasis. Tetraspanins and cholesterol are co-enriched in specific microdomains (Charrin et al., 2003). Such localization can be attributed to the palmitoylation of tetraspanins that drives their affinity for cholesterol enriched domains, alternatively the crystal structure of the tetraspanin CD81 has revealed the existence of an intramembrane cavity in which cholesterol can be accommodated (Zimmerman et al., 2016). Therefore, we hypothesized that CD63 could also regulate cholesterol trafficking and hence its sorting to exosomes. In order to label cholesterol, we used the D4 fragment of perfringolysin O ( $\theta$  toxin, *Clostridium perfringens*) fused to GFP (D4-GFP) (Shimada et al., 2002; Wilhelm et al., 2017). To avoid the presence of cholesterol containing LDL cholesterol in endosomes that would hinder specific detection of cholesterol on ILVs, WT or CD63 KO HeLa cells were grown in lipoprotein depleted serum (LPDS)

supplemented media for at least two days before recovering exosomes and process cells for EM analysis. EM immunogold labeling of cholesterol using D4-GFP on exosomes derived from HeLa grown in LPDS (Fig. 2A) showed a reduction of cholesterol content in CD63 KO exosomes (Fig. 2B), suggesting that CD63 regulates the sorting of cholesterol to exosomes. Similar observations were made when cells were grown in FBS, (i.e. in presence of serum-derived LDL cholesterol), suggesting that CD63 would contribute to cholesterol sorting into ILVs, independently of the cholesterol origin (Fig. S2A and S2B). A previous report using perfringolysin O for immune-electron microscopy identified ILVs in MVEs as the main site of cholesterol localization and suggested the enrichment in cholesterol content as a feature of “secretory” MVEs (Möbius et al., 2003). In agreement with this report, we could identify CD63-positive MVEs containing cholesterol in ILVs in WT cells, where cholesterol and CD63 co-localize in ILVs (Fig. 2C), both in the case of cells grown in LPDS (Fig. 2C) and in the case of cells grown in FBS (Fig. S2C). In CD63 KO cells cholesterol staining on ILVs was largely decreased (Fig. 2C and 2D) in line with the decrease observed on exosomes.

Finally, we observed MVEs completely devoid of cholesterol (Fig. 3A), supporting the idea that subpopulations of MVEs exist.

### **Absence of CD63 promotes cholesterol retrieval from MVEs toward Golgi apparatus**

Surprisingly, cholesterol did not accumulate on the delimiting membrane of the MVEs in absence of CD63 (Fig. 3A, 2C and 3B), suggesting that if not sorted to ILVs cholesterol can be retrieved from the endosomal system and be transported to other compartments. Supporting this hypothesis, cholesterol staining was highly increased in the Golgi apparatus (Fig. 3A and 3B) while we could not observe any accumulation of cholesterol at the plasma membrane (Fig. S2D). In addition, we observed a trend for cholesterol enrichment in tubular/vesicular structures emanating from MVEs in CD63 KO cells (Fig. 3B), that likely support recycling of cargoes from MVEs. Of note, our EM analysis did not reveal any increase of membrane contact sites between MVEs and the ER. These data suggested that, in absence of CD63, cholesterol is not targeted to ILVs and targeted from the endosomal system to the Golgi apparatus through vesicular retrograde transport.

To support the possibility that the absence of CD63 induced cargo retrieval from endosomes by retrograde transport, we first performed EM analysis of HeLa cells WT and CD63 KO in order to analyze the morphology of MVEs and tubular/vesicular carriers. MVEs in HeLa CD63 KO cells presented a higher number of emanating tubules and a higher number of budded structures in close proximity of these MVEs (Fig. 3A and 3B), suggesting that formation of tubular and vesicular carriers for cargo recycling is stimulated in absence of CD63. We then monitor the retrograde transport of the cation-independent mannose 6-phosphate receptor (CI-M6PR), a transmembrane protein that cycles between endosomes and Trans-Golgi network and is also present at the plasma membrane at lower levels. After 1-hour internalization of an antibody anti-CI-M6PR, CI-M6PR was primarily localized in the Golgi complex in WT HeLa cells, while it was mostly dispersed in peripheral vesicles in CD63 KO

cells (Fig. 3C and 3D), indicating that retrograde transport of CI-M6PR from endosomes to Golgi was impaired and delayed.

### **Formation of endosomal vesicular carriers requires Arp 2/3 dependent actin polymerization**

Polymerization of actin on endosomal membranes contributes to many processes such as endosomal maturation and ILV formation, sorting of cargoes in specific membrane domains and maintenance of tubular domains for the recycling of endosomal cargoes (Simonetti and Cullen, 2019). Given the increased tubulation and budding observed in CD63 KO cells, we assessed a potential modulation of endosomal actin polymerization in absence of CD63. SR-IFM analysis of actin stained with phalloidin showed an accumulation of actin around EEA1 positive early endosomes (Fig. 4A and 4B). This accumulation was lost in CD63 KO cells but not in WT cells when cells were treated with the Arp 2/3 inhibitor CK666 (Fig. 4C and Fig. S3A-B) showing that absence of CD63 induced branched actin accumulation at MVEs. Finally, EM analysis of CK666 treated cells showed the presence of long tubules emanating from multivesicular endosomes in CD63 KO HeLa cells, suggesting that actin polymerization may be required for tubule maintenance and scission of cholesterol enriched buds (Fig. 4D and 4E).

### **CD63 regulates cholesterol trafficking and the formation of endosomal tubular/vesicular carriers in pigmented melanoma cells**

To investigate the physiological relevance of CD63 dependent cholesterol sorting to ILVs and exosomes we refined our previous reports on the role of CD63 in the ESCRT-independent sorting of the melanosomal protein PMEL (van Niel et al., 2011) and ApoE (van Niel et al., 2015) to ILVs and exosomes and their contribution to pigmentation in melanocytes.

MNT-1 cells were treated with Ctrl siRNA or CD63 siRNA and grown in LPDS supplemented media. Alternatively, CD63 KO cells generated using CRISPR/CAS9 technique were used.

As expected, ApoE and cholesterol co-localized in MVEs/ Stage I melanosomes of WT MNT-1 cells, where CD63 is involved in cargo sorting to ILVs, of WT MNT-1 cells and were both sorted into ILVs (Fig. 5A). Moreover, exosomes were isolated from MNT-1 WT or CD63 KO (Fig. 5B) and stained with D4-GFP. As for HeLa cells, we observed a decrease in cholesterol content of exosomes isolated from CD63 KO cells (Fig. 5c and 5D). ILVs in stage I melanosomes, were also devoid of cholesterol in CD63 siRNA treated MNT-1 cells. On the contrary, cholesterol was enriched in Golgi apparatus (Fig. 5E). EM analysis after high pressure freezing of CD63 siRNA treated MNT-1 cells showed impaired PMEL17 amyloid fibril formation in early melanosomes, as previously described (van Niel et al., 2011), and the presence of tubules and budded structures in close proximity of these endosomes, as in the case of HeLa (Fig. 6A). In addition, the retrograde transport of CI-M6PR was also perturbed (Fig. S4A and S4B). Finally, SR-IFM analysis of actin stained with phalloidin showed an accumulation of actin around EEA1 positive Stage I melanosomes (Fig. 6B and 6C).

### **CD63 regulates the intracellular trafficking of ApoE between ILVs and Golgi apparatus in pigmented melanoma cells**

Floatation of MNT-1 100,000 g pellet on a bottom-up density gradient identified different exosome-containing fractions between 1,08 g/ml and 1,17 g/ml (Fig. 7A). Exosomal markers (CD63, CD9, alix and syntenin) were particularly enriched in fraction 6/7 (corresponding to a density of 1,09-1,10 g/ml) and in fraction 9 (corresponding to a density of 1,12 g/ml) (Fig. 7B and Fig. S4C). Surprisingly ApoE was enriched only in fraction 9 (Fig. 7A and 7B), suggesting that ApoE positive exosomes represent a specific subpopulation of exosomes secreted by MNT-1 cells but not by HeLa cells. EM analysis of the two fractions showed the presence of smaller particles in fraction 9 (Fig. 7C and Fig. 7D). As quantified on EM pictures, exosomes floating in fraction 6/7 had a mean diameter of 80 nm (68% of the exosomes were in the range 50nm-100nm), while exosomes in fraction 9 had a mean diameter of 40 nm (79% of exosomes were smaller than 50 nm) (Fig. 7D). This heterogeneity would suggest the co-existence of different ILV subpopulations that may arise from distinct compartments.

We then investigated exosome secretion in Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS supplemented media. EVs from the same number of Ctrl or CD63 siRNA treated MNT-1 cells were isolated by differential ultracentrifugation and the 100,000 g pellet was recovered and analyzed by WB. As observed in HeLa cells, the levels of the exosomal markers CD9, alix and syntenin were not significantly changed (Fig. 8A and Fig. 8B). Both Ctrl and CD63 siRNA treated MNT-1 cells released a similar number of exosomes as shown by NTA analysis (Fig. S4D) and size distribution was also not affected (Fig. 8C). As we had previously reported (van Niel et al., 2015), levels of ApoE in exosomes derived from CD63 siRNA treated cells were reduced (Fig. 8A and Fig. 8B).

Cellular content of CD9, alix and syntenin was not changed while we observed a trend for increased levels of ApoE, even if the difference was not significant (Fig. 8D and Fig. 8E). These data suggest that depletion of CD63 did not affect global exosome secretion, but it regulated the specific sorting of ApoE to exosomes. SR-IFM analysis of ApoE cellular localization showed an accumulation of ApoE in TGN in CD63 depleted cells (Fig. 8F and Fig. 8G). Increased co-localization was also observed between ApoE and the cis-Golgi marker GM-130 (Fig. S4F and Fig. S4G). On the contrary, we observed low co-localization of ApoE with the lysosomal marker Lamp1 after depletion of CD63 excluding its targeting to lysosomes (Fig. S4H and Fig. S4I). Overall, these data suggest that ApoE did not undergo lysosomal degradation in absence of CD63, but was transported to the TGN and Golgi apparatus, in a similar manner than cholesterol.

## Discussion

Our data show that the absence of CD63 does not affect global exosome secretion in both HeLa and MNT-1 cells, as previously reported in other cell types such as WT fibroblasts (Gauthier et al., 2017). Yet, CD63 expression has been shown to regulate exosome secretion in fibroblasts from Down syndrome's model mice (Gauthier et al., 2017). In a similar manner, depletion of CD63 does not affect ILV formation in HeLa cells, as shown here and in a previous report (Edgar et al., 2014), while it reduced the generation of exosomes harboring ApoE (van Niel et al., 2015) and ILVs harboring PMEL in the melanocytic cell line MNT-1 cells (van Niel et al., 2011). These previous reports suggested that CD63 function may be limited to the sorting of a particular cargo or the generation of a given subpopulation of ILVs, which both would be cell-type and cell-condition dependent. In this study we showed that CD63 regulates the sorting of cholesterol into ILVs and exosomes, establishing the first molecular mechanisms that support the active sorting of a lipid to exosomes. The capacity of CD63 to sort cholesterol into ILVs is in line with the observation that the trafficking of several CD63 dependent cargoes is linked to cholesterol levels. This is the case of ApoE, which mostly associates to cholesterol containing lipoproteins mediating cholesterol transport. Moreover, cargoes such as MHC-II, whose exosomal secretion is modulated by CD63 (Petersen et al., 2011), contain a cholesterol binding domain (Roy et al., 2013), while cargoes such as Synaptotagmin VII or LMP1 contain palmitate moieties (Flannery et al., 2010; Verweij et al., 2015) which, by interacting with cholesterol, may stabilize their localization in CD63 and cholesterol enriched domains. It is therefore tempting to propose that the diverse roles of CD63 in distinct cell types could be unified by a central role in the regulation of cholesterol trafficking.

Overall, our data suggest that CD63 would generate microdomains enriched in cholesterol and partner cargoes at endosome delimiting membranes that undergo inward budding to generate cholesterol enriched ILVs. Although this clustering step could contribute to initial membrane bending, it is not sufficient to complete ILV formation. In line with this hypothesis, reported exosome biogenesis pathways, such as the ESCRT-dependent pathway and the syntenin-alix pathway, are not impaired by CD63 depletion (Baietti et al., 2012; Edgar et al., 2014). Hence, complete ILV formation would require additional machinery. Syntenin-alix pathway represent a good candidate, considering that CD63 is a direct interactant of syntenin (Latysheva et al., 2006) and that depletion of syntenin impairs the biogenesis of CD63 positive vesicles both *in vitro* and *in vivo* (Baietti et al., 2012; Verweij et al., 2019).

How does CD63 regulate the sorting of cholesterol into ILVs? CD63, as all the tetraspanins, organizes as dynamic nanoclusters in a cholesterol dependent manner within tetraspanin enriched microdomains (Charrin et al., 2003). Alternatively, CD63 may allocate a cholesterol moiety in between its transmembrane domain, as recently shown for CD81, another tetraspanin also found in exosomes (Zimmerman et al., 2016). CD63 passes through the PM (Pols and Klumperman, 2009), hence, we cannot exclude that CD63 has similar roles at the PM regulating the sorting of cholesterol during budding of shed microvesicles, especially

considering the abundance of cholesterol in shed microvesicles (Del Conde et al., 2005). Alternatively, a similar role may be played by other tetraspanins such as CD81 or CD9, although no compensation was observed at MVEs in absence of CD63.

The preferential secretion of ILVs enriched in cholesterol as exosomes (Möbius et al., 2003) suggested that sorting of cholesterol at MVEs would be of prime importance to regulate the fate of MVEs between lysosomal degradation or secretion (van Niel et al., 2018). High concentration of cholesterol on the delimiting membrane of endosomes has been shown to cluster late endosomes in the perinuclear region and to target them for lysosomal degradation (van der Kant et al., 2013; Rocha et al., 2009). While cholesterol export by vesicular and non-vesicular transport offers a first level of regulation of endosomal cholesterol levels, CD63 would provide an additional pathway of regulation by redistributing cholesterol from the delimiting membrane to ILVs. Surprisingly, our data show that in absence of CD63, cholesterol is not retained on the delimiting membrane of endosomes. In line with this observation, we do not observe endosomal accumulation of cholesterol, as it is observed when NPC1 function are impaired, and we do not observe perinuclear accumulation of endosomes, as we would expect if cholesterol was accumulated on the delimiting membrane. We observed instead an increase of tubular/vesicular carriers emanating from endosomes, suggesting that the defect of cholesterol sorting to ILVs is counterbalanced by its retrieval from MVEs.

Interestingly we found that cholesterol tended to be enriched in carriers in absence of CD63, suggesting that cholesterol egresses the endosomal system through vesicular transport. Although we could not identify the molecular machinery responsible for cholesterol egress, we have previously shown that depletion of CD63 in melanoma cells increase the number of flat clathrin coat containing the ESCRT-0 component HRS (van Niel et al., 2011). HRS have been involved in the egress of cholesterol from endosomes, despite the molecular mechanism has not been identified yet (Du et al., 2012). As component of the ESCRT complex, HRS participate to the biogenesis of a subpopulation of ILVs with different size from the CD63-dependent ILVs (Edgar et al., 2014), hence contributing to cargo degradation. In addition, a direct interaction of HRS with the retromer proteins Vps35, SNX1 and SNX3, have been reported, involving HRS in the retrograde transport of cargoes (Pons et al., 2008; Popoff et al., 2009). Therefore, HRS seems to contribute to maintain a balance between degradative pathway and recycling pathway. A recent study reported that HRS can promote the constitutive recycling of cargo proteins, including CI-M6PR, through the recruitment of the actin nucleator factor WASH (MacDonald et al., 2018), which is also a known interactant of the retromer complex (Harbour et al., 2012). Supporting this hypothesis, we found that depletion of CD63 induced the accumulation of actin patches on endosomal membranes. Actin polymerization was dependent on the Arp 2/3 complex and chemical inhibition of the complex induced the formation of long tubules emanating from endosomes, indicating that actin is required for the stabilization and release of recycling tubular carriers, as previously proposed (Bissig et al., 2019; Puthenveedu et al., 2010; Ripoll et al., 2018). Surprisingly, we found that the retrograde trafficking of CI-M6PR, whose recycling is also dependent on actin

dynamics and on the retromer pathway among others, was delayed. Interestingly, it has been shown that normal levels of free cholesterol are required for CI-M6PR egress from endosomes (Miwako et al., 2001). Hence, one possibility is that alteration in cholesterol levels and availability in endosomes induced by the absence of CD63 would in turn impair CI-M6PR recycling. A stimulation of the retrograde transport in absence of CD63 is supported by the enrichment of cholesterol in the Golgi apparatus in CD63 knock out cells. This localization could represent the final destination or a step in the trafficking of cholesterol to other cellular compartments. Indeed, several studies have suggested that cholesterol can move from late endosomes to the TGN prior to its transfer and esterification in the ER or to its delivering to the plasma membrane (Garver et al., 2002; Urano et al., 2008). However, the constant accumulation of cholesterol in Golgi is in favor of the first hypothesis.

Confirming our previous study (van Niel et al., 2015), we showed that CD63 regulates the sorting of ApoE to ILV and exosomes in the pigment melanoma cell line MNT-1. In absence of CD63 ApoE is accumulated in the Golgi apparatus and Trans-Golgi Network but not in lysosomes, contrary to the other CD63 dependent cargo PMEL (van Niel et al., 2011), suggesting that CD63 not only regulates the balance between ILV sorting and lysosomal degradation but also a balance between ILV sorting and retrograde transport. Very little is known about the intracellular trafficking of ApoE with most of studies being related to macrophages. It has been proposed that a pool of ApoE fated for secretion and a pool fated for degradation coexists and that ApoE can undergo recycling to the plasma membrane and to the Golgi apparatus (Kockx et al., 2008). ApoE released through exosomes could then constitute an alternative way of secretion or a way to avoid lysosomal degradation. Regardless of its origin, an endosomal pool of ApoE is required for the formation of physiological PMEL amyloid fibrils in pigment cells (van Niel et al., 2015). Here we showed that CD63 regulates not only the sorting of ApoE but also of cholesterol. While current evidence indicates that cholesterol levels and membrane lipid composition can modulate the formation of toxic A $\beta$  amyloid fibrils (Grimm et al., 2017), the possible influence of cholesterol on PMEL amyloid fibril formation is not known. Beside a role for cholesterol, in concert with CD63, in the formation of microdomains in which PMEL is partially cleaved before its sorting to ILVs, cholesterol may contribute to create specific platforms required for PMEL fibril formation at the surface of ILVs (van Niel et al., 2015).

Finally, we showed that secretion of both ApoE and cholesterol on exosomes are regulated by CD63. Being ApoE-lipoproteins major carriers of cholesterol, ApoE positive exosomes may represent a new pathway of cholesterol efflux from cells. Such pathway may be of great importance in pathological condition such as Niemann-Pick type C disease, in which cholesterol accumulation in late endosomes/lysosomes is ameliorated by the release of cholesterol-rich exosomes (Strauss et al., 2010). In addition, the presence of cholesterol and ApoE on exosomes would also extend or modify the properties of these exosomes, that could be taken up by recipient cells, through interaction with specific ApoE receptors, providing a new source of cholesterol to recipient cells.

## Materials and methods

### Cell culture, transfection and drug treatments

HeLa cells were cultured in DMEM supplemented with 10% (v/v) FBS, penicillin-streptomycin (Gibco). MNT-1 cells were maintained in DMEM supplemented with 20% FBS, 10% AIM-V medium, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin (Gibco). Where indicated, FBS was substituted by LPDS 1/30 (Millipore). All cells were maintained at 37°C in a 5% (v/v) CO<sub>2</sub> incubator. For siRNA transfection, MNT-1 cells were seeded at day 1, transfected with siRNAs (200 pmol) at day 3 and at day 5 using Oligofectamine (Thermo Fisher Scientific), according to the manufacturer's instructions, and analyzed at day 7. CK666 (Tocris Biosciences) treatments were for 2 hours at 200 μM.

### Generation of CRISPR/CAS9 CD63 KO

CD63 KO HeLa cells or MNT-1 cells were generated using CRISPR/Cas9 gene editing technology. The target sequence was selected using the CRISPR design tool available at the Broad Institute (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design>). The target sequence is: CCAGTGGTCATCATCGCAGT. The corresponding guide DNA sequences were cloned into the lentiCRISPRv2 plasmid (Addgene #52961) according to the instructions of the Zhang laboratory (<http://www.genome-engineering.org/gecko/>) (Ran et al., 2013). The plasmid was transfected using FuGENE HD according to the manufacturer's instructions for HeLa cells and the Neon Electroporation Transfection System (Thermo Fisher Scientific) for MNT-1 cells. Cells were treated after 36-48h with 10 μg/ml puromycin for 36-48h. Cells negative for CD63 were sorted following staining with the CD63 mAb TS63 using a FACS Aria cell sorter (Beckton Dickinson). CD63 KO HeLa clones #1 and #2 were obtained by single-cell culture in 96-well plates.

### Reagents, antibodies and siRNAs

Reagents were obtained from the following sources: D4-GFP was a kind gift of Fabien Alpy (IGBMC, Illkirch, France), phalloidin conjugated to Alexa Fluor 647 from Thermo Fisher Scientific.

Antibodies were obtained from the following sources: anti-CD63 (CLB-180, ab23792, 1:200 dilution for WB, IF and EM), anti-CD9 was a kind gift of Eric Rubinstein (Inserm, U935, Villejuif, France), anti-alex (pab0204, Covalab, 1:500 dilution for WB), anti-syntenin (ab19903, dilution 1:500 for WB), anti-tubulin (ab6046, 1:10,000 dilution for WB), anti-β-actin (A5316, Sigma, 1:2000 dilution for WB), anti-ApoE (ab52607, dilution 1:500 for IF and WB), anti-EEA1 (ab70521, dilution 1:200 for IF), anti-TGN46 (AHP500GT, Biorad, dilution 1:300 for IF), anti-GM130 (610822, BD Transduction Laboratories, dilution 1:1000 for IF), anti-CI-m6PR (ab8093, dilution 1:100 for pulse/chase), anti-Lamp1 (555798, BD Biosciences, dilution 1:200 for IF), anti-GFP (A11122, dilution 1:400 for EM), secondary anti-rabbit IgG or anti-mouse IgG or anti-sheep IgG antibodies conjugated to Alexa Fluor 488, 555 or 647 from Thermo Fisher Scientific and used 1:200, HRP-conjugated goat anti-mouse, anti-rabbit from

Abcam and used 1:10,000; protein A conjugated to 5 or 10 nm gold particles (PAG; 1:50; Cell Microscopy Center, Utrecht University Hospital, Utrecht, Netherlands).

The sense strand for the indicated double-stranded siRNAs were synthesized with the following sequences (Qiagen): siCtrl, 5'-AATTCT CCG AAC GTG TCA CGT-3'; siCD63, 5'-AAGTTCTTGCTCTACGTCCTC-3'.

### **Exosome isolation and characterization**

Exosomes were prepared from conditioned media incubated for 48 hr on sub-confluent cells. FBS supplemented medium was previously centrifuged at 100,000 g for 16 hours to remove FBS-derived exosomes. Conditioned media were centrifuged at 300 g for 15 min (4° C) and 2000 g (20 min, 4°C) to remove cell debris. Next, the supernatant was centrifuged at 10,000 g for 30 min (4°C) and exosomes were collected from the supernatant by centrifugation at 100,000 g for 60 min (4°C, 45Ti or 70Ti rotor, Beckman Coulter). The pellet was washed in PBS (pH 7) by centrifugation at 100,000 g for 60 min (4°C) and finally resuspended in PBS (pH 7). Bottom-up density gradient was performed as previously described (Van Deun et al., 2014) with some modifications. Solutions of 5, 10, 20 and 40% iodixanol were made by mixing a homogenization buffer [0.25 M sucrose, 1 mM EDTA, 10 mM Tris-HCL, (pH 7.4)] and an iodixanol working solution, prepared by combining a working solution buffer [0.25 M sucrose, 6 mM EDTA, 60 mM Tris-HCL, (pH 7.4)] and a stock solution of OptiPrep™ (60% (w/v) aqueous iodixanol solution, Sigma-Aldrich). Briefly, 100,000 g pellets were mixed with the bottom fraction to obtain a 40% fraction. The gradient was formed by layering 40%, 20%, 10% and 5% solutions on top of each other in an open top polypropylene tube (Beckman Coulter). The gradient was then centrifuged for 14 hours at 100,000 g (4°C, SW41 rotor, Beckman Coulter). Gradient fractions of 1 mL were collected from the top of the gradient, diluted in PBS and centrifuged for 1 hour at 100,000 g (4°C). The resulting pellets were resuspended in PBS (pH 7). For D4-GFP staining on exosomes, conditioned media were first centrifuged at 300 g for 15 min (4° C) and 2000 g for 20 min (4°C) to remove cell debris and concentrated by ultrafiltration on a 9,000 MWCO filter (Thermofisher) to obtain a concentrated conditioned media (CCM). CCM was then incubated with D4-GFP (dilution 1:1000) overnight at 4°C under rotation and fixed with 2% PFA for 15 min. Exosomes were then isolated by size exclusion chromatography (IZON Science). Fraction from 7 to 9 were recovered, pulled and concentrated again using a 10,000 MWCO filter (Centricon, Merck-Millipore). These samples were then processed for electron microscopy as described below.

### **Nanoparticle-tracking analysis**

NTA measurements of EV size and concentration were performed using a ZetaView®, Particle Metrix. Samples were pre-diluted with filtered PBS and measured at 11 different position for 2 cycles at 24°C. The videos were then processed by the ZetaView Software 8.04.02 SP.

### **Western Blot**

Cells were lysed on ice in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, and 1 mM EDTA, pH 7.2) supplemented with a protease inhibitor cocktail (Roche). Cell lysates or exosomes were incubated with sample buffer with or without 350 mM 2-mercaptoethanol (Sigma), incubated at 60°C for 30 min, loaded on 4–12% Bis-Tris gels (Nu-PAGE, Invitrogen), and transferred on nitrocellulose membranes (GE Healthcare). Membranes were blocked in PBS/0.1% Tween (PBS/T) with 5% nonfat dried milk, incubated with indicated primary and secondary antibodies diluted in PBS/T-milk. Western blots were developed using the ECL SuperSignal West Pico or Dura (Thermo Fisher Scientific). The presented immunoblots are representative of at least three independent experiments.

### **IF and super-resolution microscopy**

Cells were grown on coverslips and fixed for 20 minutes at RT with 4% PFA/PBS and quenched for 10 min with PBS/ 50 mM glycine. Cells were incubated with PBS/ 0.2% BSA/ 0.1% saponin before incubation with primary and secondary antibody in the same buffer. Incubation with phalloidin AF-647 was performed during the secondary antibody incubation. For ApoE staining, cells were fixed with cold methanol for 5 min at -20°C and IF was performed using PBS/ 0.2% BSA. Cells were washed using the same buffer and coverslips were mounted using Prolong Gold antifade reagent with DAPI (Invitrogen). Staining of plasma membrane cholesterol was performed using D4-GFP as previously described (Wilhelm et al., 2019) and images were acquired using a confocal microscope Leica SP8 equipped with a 63x 1.4 NA objective.

For super-resolution microscopy cells were analyzed as previously described (Ripoll et al., 2018). Briefly, images were acquired on a spinning disk system (Gataca Systems) based on an inverted microscope (Ti-E; Nikon) equipped with a sCMOS camera (Prime 95B; Photometrics), a confocal spinning head (X1; Yokogawa), a 100x 1.4 NA Plan-Apo objective lens, and a superresolution module (Live-SR; Gataca systems) based on structured illumination with optical reassignment technique and online processing leading to a two-time resolution improvement.

Alternatively, coverslips were analyzed on a wide-field microscope (Eclipse 80i Upright; Nikon) equipped with a CCD camera (CoolSNAP HQ2; Photometrics) using a 100x 1.4 NA Plan-Apo objective lens. Z images series were acquired every 0.2  $\mu\text{m}$  with the piezoelectric motor (Physik Instrumente) using MetaMorph software (Molecular Devices). Raw images were deconvolved with Meinel algorithm (PICT-IBISA imaging platform at Institut Curie).

### **EM**

For conventional electron microscopy, cells were seeded on coverslips and fixed with 2.5 % glutaraldehyde in 0.1 M cacodylate buffer. Cells were processed for Epon (TAAB Laboratories Equipment) embedding as described (Raposo et al., 2001) and ultrathin sections were contrasted with uranyl acetate and lead citrate.

For high-pressure freezing, cells were cultured on carbonated sapphire disks, high-pressure frozen with HPM Live  $\mu$  (CryoCapCell) and then freeze substituted in anhydrous acetone containing 1% OsO<sub>4</sub>/2% H<sub>2</sub>O for 64 h in a freeze substitution system (AFS; Leica Microsystems). Cells were embedded in Epon and processed for sectioning and contrasting with uranyl acetate and lead citrate.

For ultrathin cryosections and immunogold labeling, cells were grown on flasks and fixed with 2% PFA/ 0.125% glutaraldehyde/ 0.1 M phosphate buffer. Cell pellets were embedded in 10% gelatin and infused in 2.3 M sucrose. Gelatin blocks were frozen and processed for ultracryomicrotomy. Ultrathin sections were immunogold labeled using PAG 5 or 10 nm as previously described (Raposo et al., 2001). For cholesterol, ultrathin sections were directly incubated for 30 min with D4-GFP (dilution 1:500), fixed with 1% glutaraldehyde and immunogold labeled using an antibody anti-GFP and PAG 10 nm.

Exosomes were directly deposited on formvar/carbon coated copper/palladium grids for 20 min at RT, fixed with PFA 2%/0.1 M phosphate buffer and processed for immunogold labeling as for cells. Negative staining of exosomes or cell ultrathin cryo-sections was performed using uranyl acetate 0.4% in methylcellulose.

The samples were analyzed with an 80 kV transmission electron microscope (Tecnai Spirit G2; Thermo Fischer, Eindhoven, The Netherlands) equipped with a 4k CCD camera (Quemesa, EMSIS, Münster, Germany).

## **Image analysis and quantifications**

### *Pearson's correlation coefficient*

Pearson's correlation coefficient between two channels was quantified using JACoP plugin of ImageJ Fiji software.

### *Phalloidin fluorescence on endosomes*

Z-stack images were acquired every 0,20  $\mu$ m. Average intensity z projections of five stacks were generated for both EEA1 and phalloidin channel. 15-pixel (1,65 nm)-diameter circles were drawn around EEA1-positive endosomes (ROI). The ROI was copied in the phalloidin channel and phalloidin fluorescence intensity in the ROI was measured.

### *Localization of internalized antibody directed against CI-M6PR*

Z-stack images were acquired every 0,20  $\mu$ m. Average intensity z projections of four stacks were generated. The total intracellular fluorescence intensity was measured by manually selecting an area covering the entire cell. The fluorescence intensity within a 7 x 7  $\mu$ m<sup>2</sup> region centered on the Golgi complex (TGN46 staining was used as reference) was then measured. The non-Golgi fluorescence (vesicular) intensity was obtained by subtracting the fluorescence intensity in the Golgi complex from the total fluorescence intensity. Data are presented as the non-Golgi/Golgi CIMPR fluorescence ratio from each cell.

### *Immunogold labeling*

Quantification of immunogold labeling on ultrathin cryosections or exosomes was performed by counting the number of gold particles per cell compartment or exosome. MVEs were defined as compartments delimited by a membrane with numerous internal

vesicles, early melanosomes were defined by morphology (Raposo et al., 2001). The count was performed on randomly selected cells.

#### *Immunoblot quantification*

Signal intensities were quantified with Image J Fiji software. Quantifications were from at least three independent experiments.

#### *Statistical analysis*

Statistical data are presented as mean  $\pm$  SEM. Statistics were calculated using unpaired Student's t test, or ordinary one-way ANOVA as specified, in GraphPad Prism 8. Significant differences between control or treated samples are indicated (\*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ). Only  $P < 0.05$  was considered as statistically significant.

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## Figure legends

### Figure 1. Characterization of EVs secreted by WT or CD63 KO HeLa cells

**A.** Western blot analysis of exosomal 100K g pellet from HeLa cells WT or CD63 KO. **B.** Quantification of exosomal protein content shown in A (3 independent experiments, unpaired multiple t-test). **C.** NTA analysis of 100K g pellet from HeLa cells WT or CD63 KO (3 independent experiments). **D.** Electron micrograph of HeLa cells WT or CD63 KO. Bars= 200 nm.

Data are shown as mean  $\pm$  SEM. \*\*\*\*,  $P < 0.0001$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ .

### Figure 2. CD63 regulates sorting of cholesterol into exosomes in HeLa cells.

**A.** Exosomes isolated from HeLa cells WT or CD63 KO grown in LPDS were observed by electron microscopy after immunogold labeling with D4-GFP (PAG 10nm). A gallery of exosomes is shown. Bars=200 nm. **B.** Quantification of the relative number of gold particles per exosome ( $n = n^\circ$  of exosomes, H WT  $n = 231$ , H CD63 KO#1  $n = 147$ , H CD63 KO#2  $n = 190$ ; 2 independent experiments, Ordinary one-way ANOVA). **C.** HeLa cells WT or CD63 KO were grown in LPDS, processed for ultrathin cryo-sectioning and immuno-gold labeled with D4-GFP (PAG 10nm) and CD63 (PAG 5nm). Bars=200 nm. **D.** Quantification of number of gold particles on intraluminal vesicles per MVE (MVEs, H WT  $n = 72$ , H CD63 KO#1  $n = 26$ , H CD63 KO#2  $n = 101$ ; 2 independent experiments, 2-way ANOVA multiple comparisons).

Data are shown as mean  $\pm$  SEM. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; ns,  $P > 0.05$ .

### Figure 3. CD63 regulates endosomal outward budding and cargo recycling

**A.** HeLa cells WT or CD63 KO were grown in LPDS, processed for ultrathin cryo-sectioning and immuno-gold labeled with D4-GFP (PAG 10nm). Black arrows indicate MVEs containing cholesterol, white arrows indicate MVEs devoid of cholesterol and arrowheads indicate Golgi apparatus. Bars=500 nm. **B.** Quantification of number of gold particles per cellular compartment (MVEs, H WT  $n = 72$ , H CD63 KO#1  $n = 26$ , H CD63 KO#2  $n = 101$ ; tubules/buds, H WT  $n = 22$  H CD63 KO#1  $n = 21$ , H CD63 KO#2  $n = 39$ ; Golgi, H WT  $n = 10$  H CD63 KO#1  $n = 11$ , H CD63 KO#2  $n = 22$ ; 2 independent experiments, 2-way ANOVA multiple comparisons). **C.** Electron micrograph of HeLa cells WT or CD63 KO analyzed by 2D EM. Bars= 200 nm. **D.** Quantification of  $n^\circ$  of tubules/buds per endosome ( $n = n^\circ$  of MVE, H WT  $n = 24$ , H CD63 KO#1  $n = 22$ , H CD63 KO#2  $n = 18$ ; 2 independent experiments, Ordinary one-way ANOVA) **E.** Anti CI-M6PR antibody was internalized for 30 minutes in HeLa cells WT or CD63 KO and IFM with secondary antibody anti-mouse for CI-M6PR. CI-M6PR staining is shown as pseudo-color. Bars= 10  $\mu$ m. **F.** CI-M6PR fluorescence intensity in the whole cells or in the Golgi area was measured. Data are shown as a ratio of vesicular fluorescence/Golgi fluorescence. ( $n = n^\circ$  of cells, H WT  $n = 51$ , H CD63 KO#1  $n = 55$ , H CD63 KO#2  $n = 52$ ; 3 independent experiments, Ordinary one-way ANOVA).

Data are shown as mean  $\pm$  SEM. \*\*\*\*,  $P < 0.0001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ .

### Figure 4. CD63 regulates Arp 2/3 dependent endosomal actin polymerization

**A.** SR-IFM of HeLa cells WT or CD63 KO costained for EEA1 and actin. Bars=10 $\mu$ m. **B.** Normalized phalloidin fluorescence on EEA1-positive endosomes ( $n = n^\circ$  of endosomes, H WT  $n = 174$ , H CD63 KO#1  $n = 228$ , H CD63 KO#2  $n = 214$ ; 3 independent experiments, Ordinary one-way ANOVA). **C.** SR-IFM of HeLa cells CD63 KO treated with DMSO or CK666 and costained for EEA1 and actin. Bars=10 $\mu$ m. **D.** Electron micrograph of HeLa cells WT or CD63 KO

treated with CK666. Bars= 200 nm. **E.** HeLa cells CD63 KO were grown in LPDS, treated with CK666, processed for ultrathin cryo-sectioning and immuno-gold labeled with D4-GFP (PAG 10nm). Bars= 200 nm.

Data are shown as mean  $\pm$  SEM. \*\*\*\*,  $P < 0.0001$ .

### **Figure 5. CD63 regulates sorting of cholesterol to ILVs in pigmented melanoma cells**

**A.** WT MNT-1 cells were grown in LPDS, processed for ultrathin cryo-sectioning and immuno-gold labeled with D4-GFP (PAG 10nm) and ApoE (PAG 5nm). Bars=200 nm. **B.** Western blot analysis of exosomes from MNT-1 cells WT or CD63 KO used for the experiment in C. **C.** Exosomes isolated from WT or CD63 KO MNT-1 grown in LPDS were observed by electron microscopy after immunogold labeling with D4-GFP (PAG 10nm). A gallery of exosomes is shown. Bars=200 nm. **D.** Quantification of the relative number of gold particles per exosome. ( $n=n^\circ$  of exosomes, M WT  $n=509$  M CD63 KO  $n=426$ ; 2 independent experiments, two-tailed unpaired t-test). **E.** Ctrl or CD63 siRNA-treated MNT-1 cells were grown in LPDS, processed for ultrathin cryo-sectioning and immuno-gold labeled with D4-GFP (PAG 10nm) and CD63 (PAG 5nm). Bars=500 nm.

Data are shown as mean  $\pm$  SEM; \*\*\*,  $P < 0.001$ .

### **Figure 6. CD63 regulates endosomal outward budding and Arp 2/3 dependent actin polymerization in pigmented melanoma cells**

**A.** Ctrl or CD63 siRNA-treated MNT-1 cells were high-pressure frozen and analyzed by 2D EM. A gallery of endosomes/stage I is shown. Bars=200 nm. **B.** SR-IFM of Ctrl or CD63 siRNA-treated MNT-1 costained for EEA1 and actin. Bars=10 $\mu$ m. **C.** Normalized phalloidin fluorescence on EEA1-positive endosomes ( $n=n^\circ$  of endosomes, M siCtrl  $n=225$ , M siCD63  $n=222$ ; 2 independent experiments, two-tailed unpaired t-test with Welch's correction). Data are shown as mean  $\pm$  SEM; \*\*\*,  $P < 0.001$ .

### **Figure 7. Characterization of EVs secreted by pigmented melanoma cells.**

**A.** Western-blot analysis of a bottom-up Optiprep density gradient of 100K g exosomal pellet from WT MNT-1 cells. **B.** Quantification of exosomal markers' distribution in fractions collected from the Optiprep density gradient as in A (3 independent experiments, 2-way ANOVA multiple comparisons). **C.** Fractions collected from Optiprep density gradient were observed by electron microscopy after negative staining. Bars=200 nm. **D.** Quantification of mean size and size distribution of exosomes as in C (3 independent experiments, two-tailed unpaired t-test).

Data are shown as mean  $\pm$  SEM. \*\*\*\*,  $P < 0.0001$ ; \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ ; \*,  $P < 0.05$ ; ns,  $P > 0.05$ .

### **Figure 8. CD63 regulates the exosomal sorting of ApoE and its intracellular trafficking in pigment melanoma cells.**

**A.** Western blot analysis of exosomal 100K g pellet from Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS. **B.** Quantification of exosomal protein content shown in A (unpaired multiple t-test,  $n= 3$  independent experiments). **C.** NTA analysis of 100K g exosomal pellet from Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS. **D.** Western blot analysis of cell lysates from Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS. **E.** Quantification of protein content shown in D (unpaired multiple t-test,  $n= 3$  independent experiments).

**F.** SR-IFM of Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS and costained for endogenous ApoE and TGN46. **G.** Pearson's correlation coefficient of cells (n = 33, two-tailed unpaired t-test) as in F.

Data are shown as mean  $\pm$  SEM. \*\*\*\*, P < 0.0001; \*\*\*, P < 0.001; \*\*, P < 0.01; ns, P>0.05.

#### **Supplementary Figure 1 (relative to Figure 1). Characterization of EVs secreted by WT or CD63 KO HeLa cells**

**A.** Western blot analysis of cell lysates from HeLa cells WT or CD63 KO. **B.** Quantification of protein content shown in A. (unpaired multiple t-test, n= 3 independent experiments). **C.** NTA analysis of 100K g pellet from HeLa cells WT or CD63 KO. **D.** Density values of the fractions recovered from bottom-up Optiprep density gradient of 100K g exosomal pellet from HeLa WT or CD63 KO cells. **E.** Western-blot analysis of a bottom-up Optiprep density gradient of 100K g exosomal pellet from HeLa WT or CD63 KO. **F.** SR-IFM of HeLa cells WT or CD63 KO stained for LAMP-1.

Data are shown as mean  $\pm$  SEM. \*\*\*\*, P < 0.0001; \*\*, P < 0.01; \*, P < 0.05; ns, P>0.05.

#### **Supplementary Figure 2 (relative to Figure 2). Effect of CD63 depletion on cholesterol trafficking.**

**A.** Exosomes isolated from HeLa cells WT or CD63 KO grown in FBS were observed by electron microscopy after immunogold labeling with D4-GFP (PAG 10nm). Bars=200 nm. **B.** Quantification of relative number of gold particles per exosome. **C.** IFM of HeLa cells WT or CD63 KO grown in LPDS and stained for cholesterol at plasma membrane using D4-GFP.

Data are shown as mean  $\pm$  SEM. \*\*\*\*, P < 0.0001.

#### **Supplementary Figure 3 (relative to Fig. 4). CD63 regulates Arp 2/3 dependent endosomal actin polymerization.**

**A.** Normalized phalloidin fluorescence on EEA1-positive endosomes. DMSO treated cells are the same shown in fig. 4B (n=n° of endosomes, H WT+CK666 n= 102, H CD63 KO#1 n=90, H CD63 KO#2 n=104; Ordinary one-way ANOVA). **B.** SR-IFM of HeLa cells WT treated with DMSO or CK666 and co-stained for EEA1 and actin. Bars=10um.

Data are shown as mean  $\pm$  SEM. \*\*\*\*, P < 0.0001; ns, P>0.05.

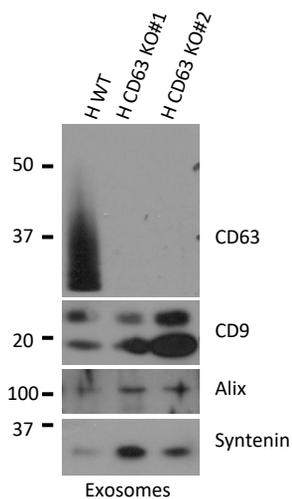
#### **Supplementary Figure 4 (relative to figure 6, 7 and 8).**

**A.** Anti CI-M6PR antibody was internalized for 30 minutes in Ctrl or CD63 siRNA-treated MNT-1 cells and IFM with secondary antibody anti-mouse (for CI-M6PR) and anti-TGN46 was performed. **B.** CI-M6PR fluorescence intensity in the whole cells or in the Golgi area was measured. Data are shown as a ratio of vesicular fluorescence/Golgi fluorescence. (M siCtrl n= 18 cells, M siCD63 n= 18 cells from 2 independent experiments, unpaired t-test). **C.** Density values of the fractions recovered from bottom-up Optiprep density gradient of 100K g exosomal pellet from MNT-1 cells. **D.** NTA analysis of 100K g pellet from HeLa cells WT or CD63 KO (unpaired t-test, 3 independent experiments). **E.** Exosomal 100K g pellets from Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS were observed by electron microscopy after negative staining. Bars=200 nm. **F.** IFM of Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS and costained for endogenous ApoE and GM130. Bars=10um. **G.** Pearson's correlation coefficient of cells (M siCtrl n = 22, M siCD63 n= 15 two-tailed unpaired t-test) as in F. **H.** IFM of Ctrl or CD63 siRNA treated MNT-1 cells grown in LPDS and costained for

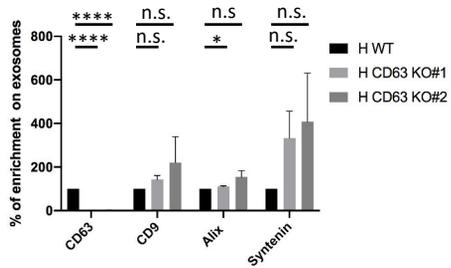
endogenous ApoE and Lamp1. Bars=10um. I. Pearson's correlation coefficient of cells (M siCtrl n= 27, M siCD63 n= 31 two-tailed unpaired t-test) as in H.  
Data are shown as mean  $\pm$  SEM. \*\*\*\*, P < 0.0001; \*\*\*, P < 0.001; \*\*, P < 0.01; \*, P < 0.05; ns, P>0.05.

Figure 1

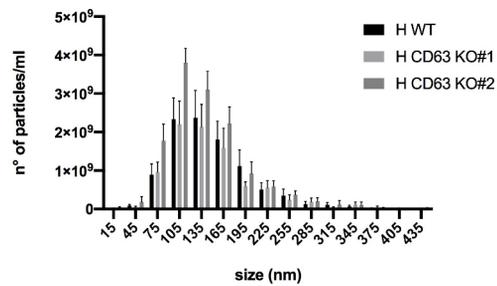
**A**



**B**



**C**



**D**

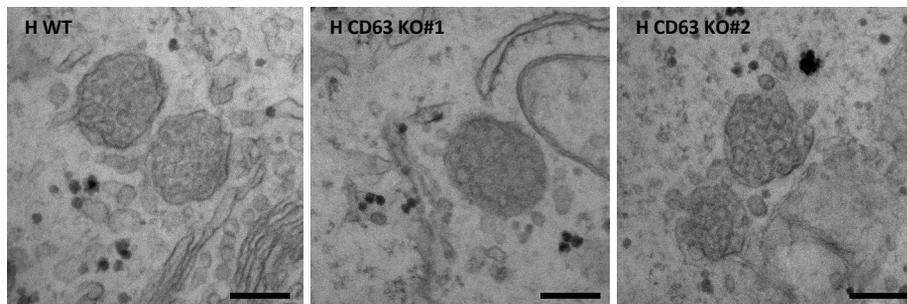
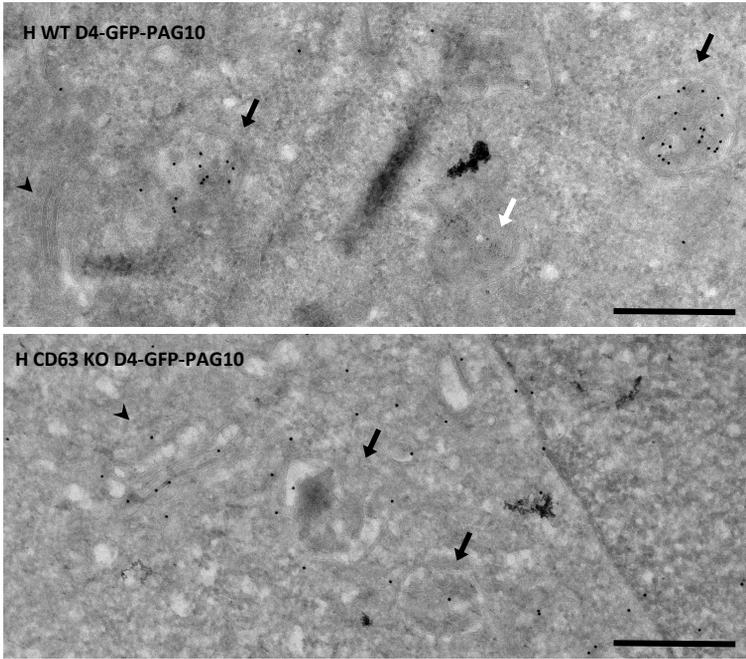


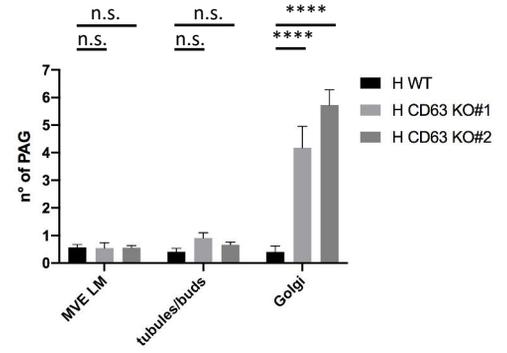


Figure 3

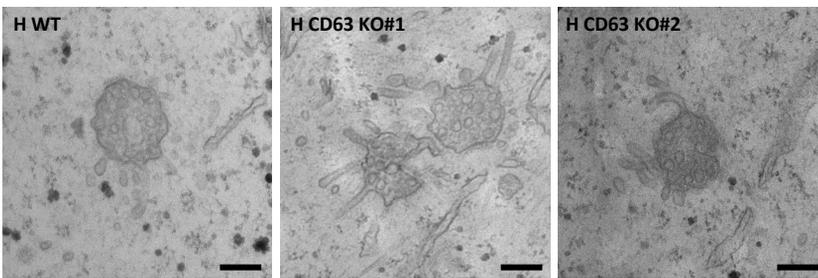
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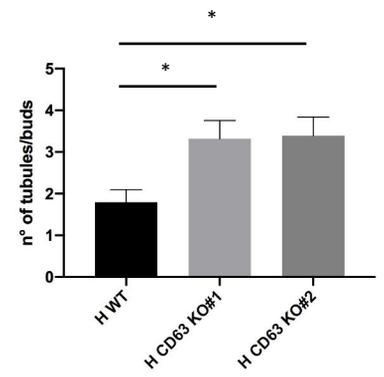
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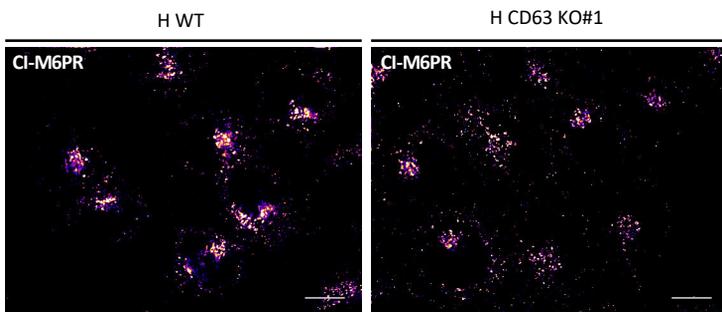
C



D



E



F

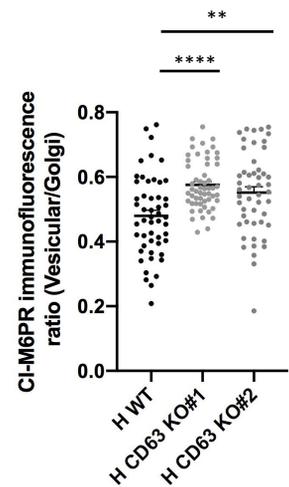




Figure 5

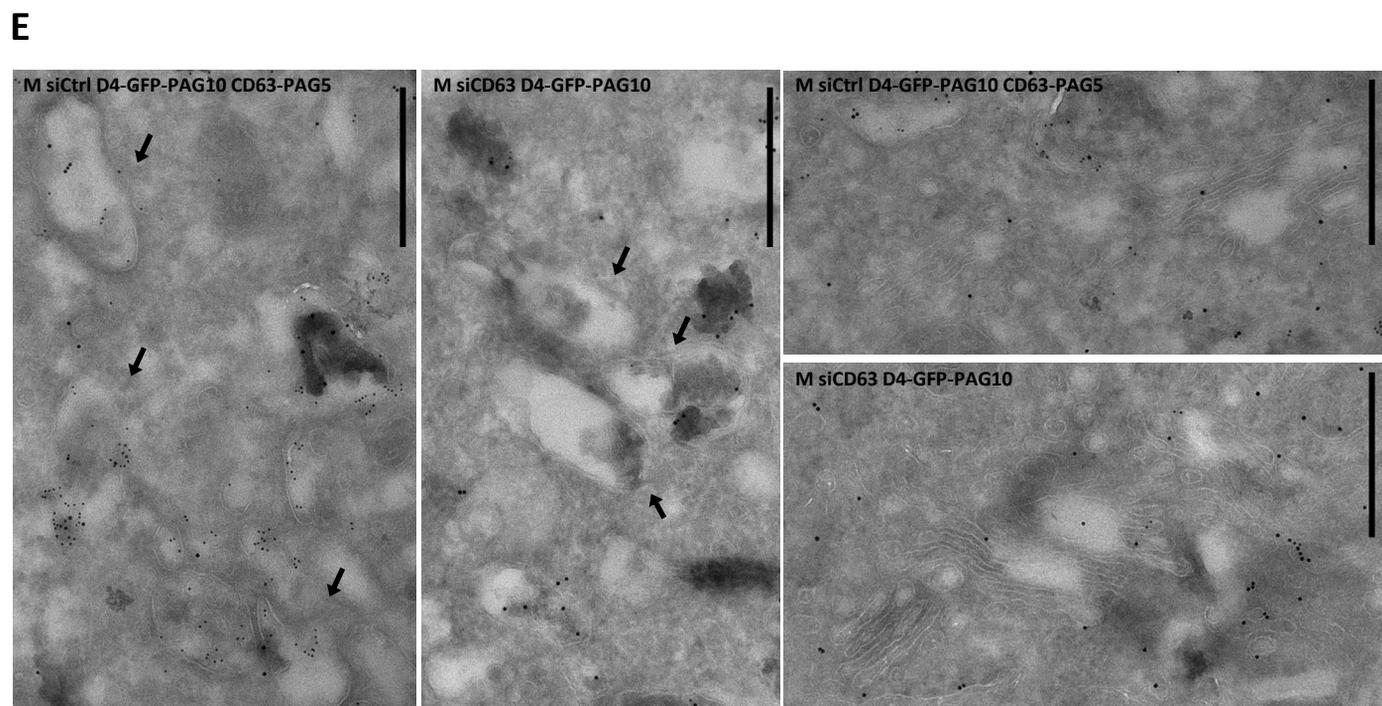
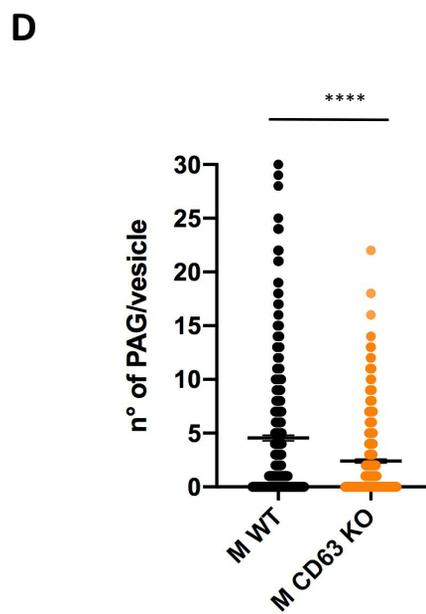
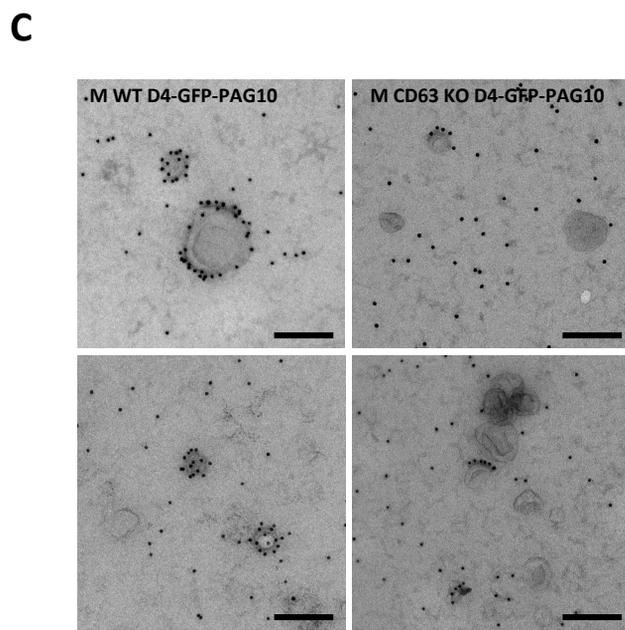
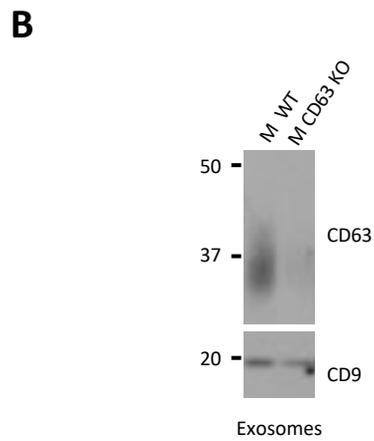
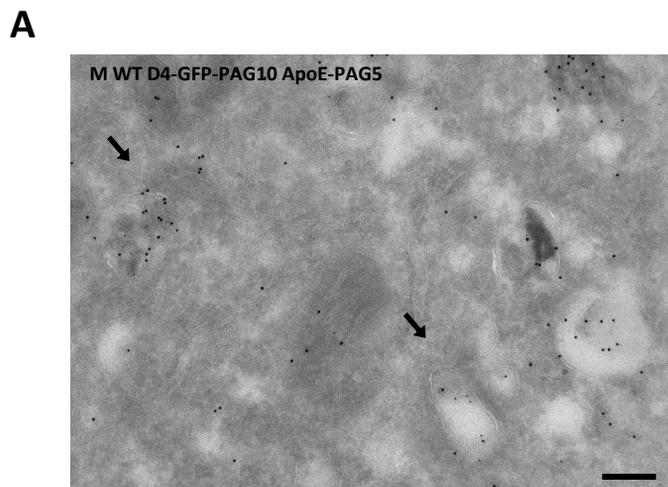
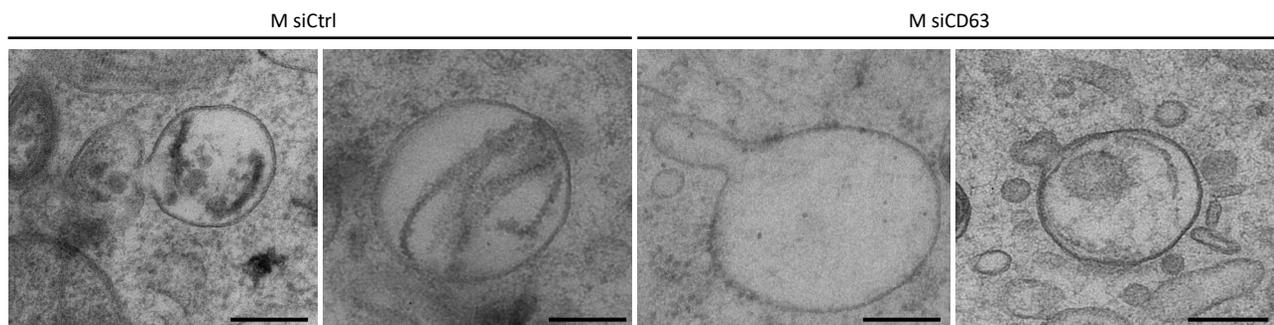
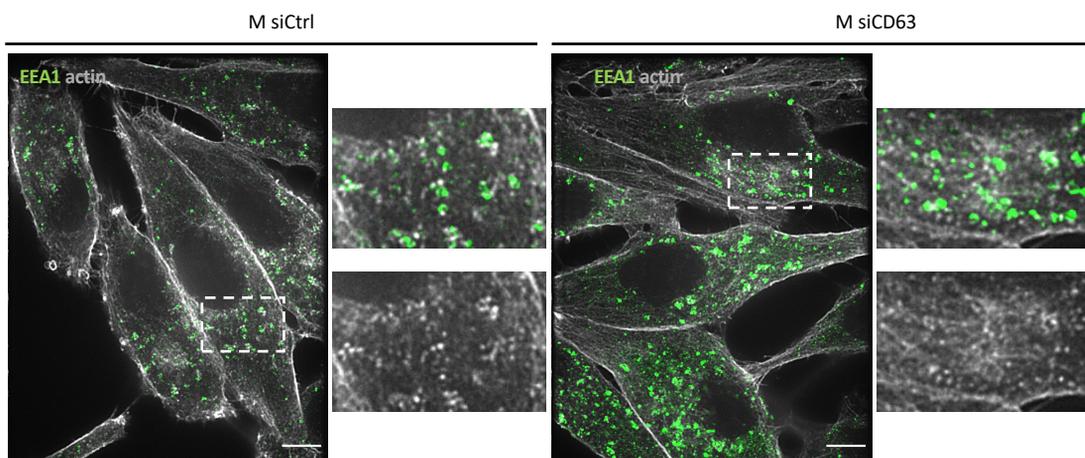


Figure 6

**A**



**B**



**C**

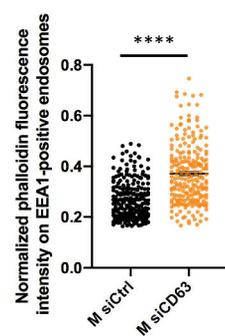
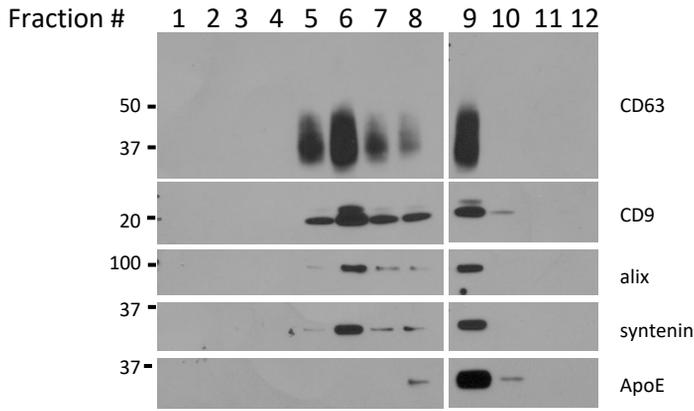
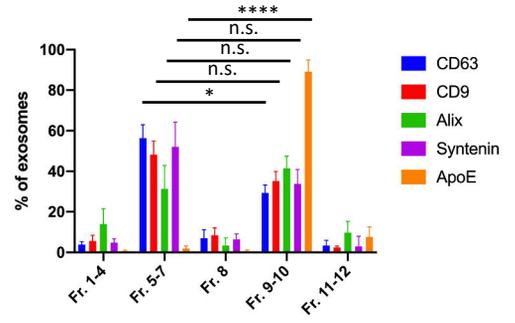


Figure 7

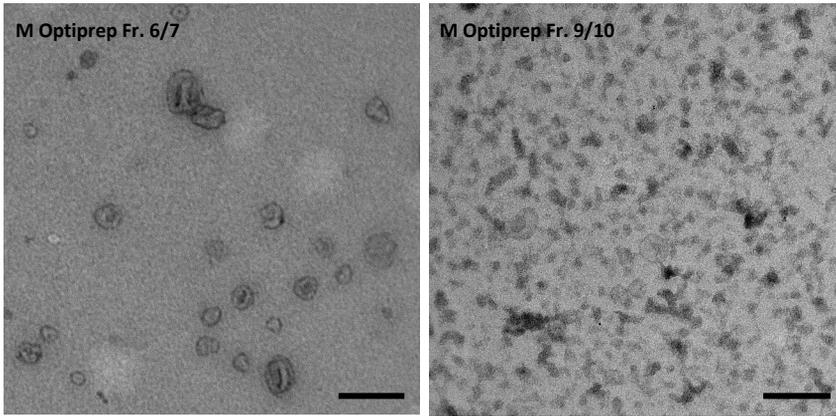
**A**



**B**



**C**



**D**

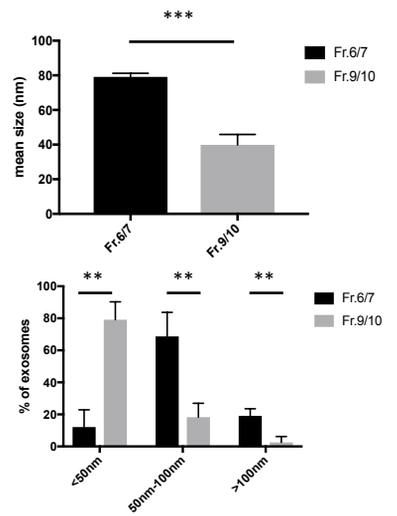
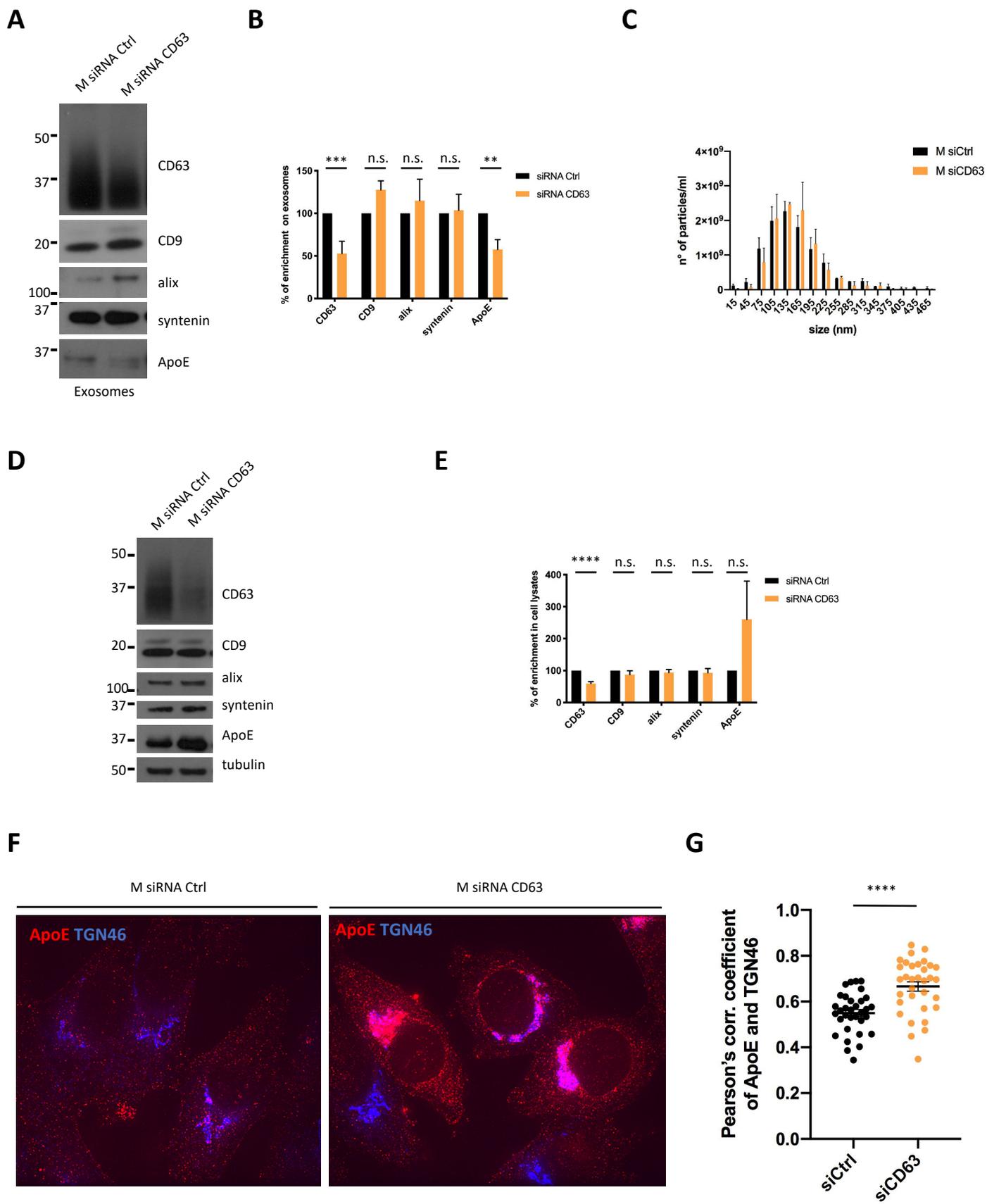
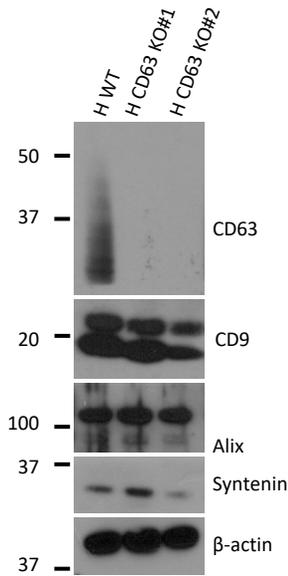


Figure 8

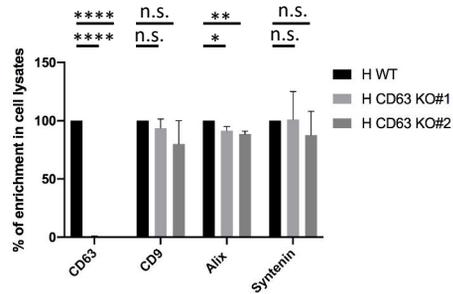


# Supplementary Figure 1

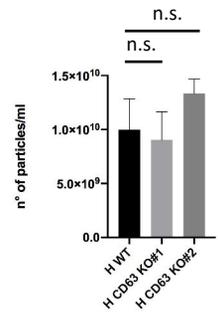
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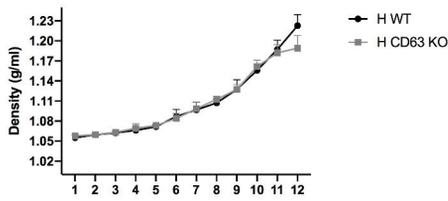
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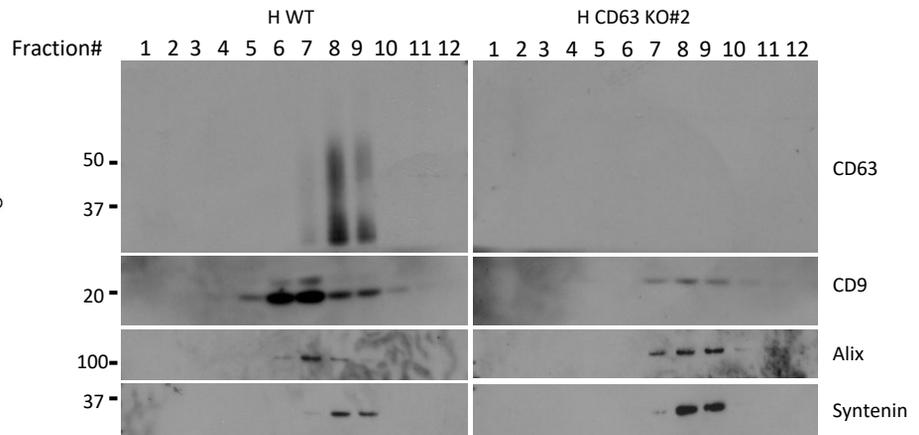
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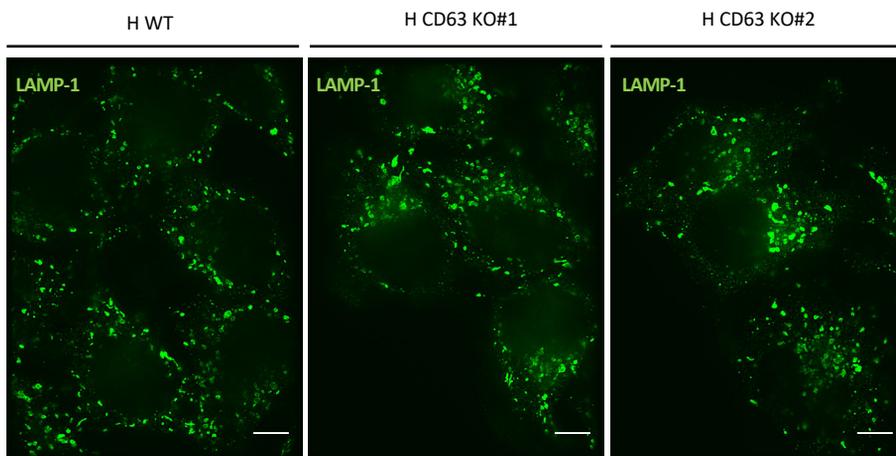
**D**



**E**

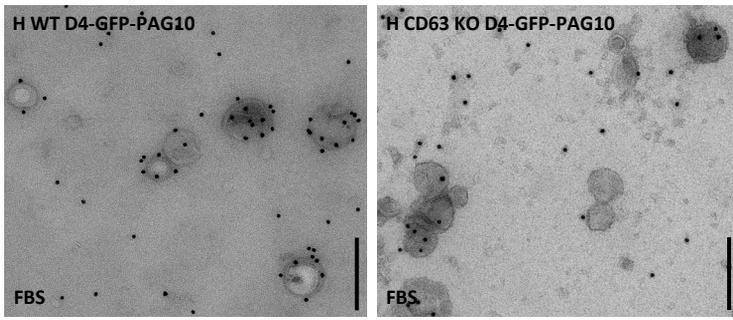


**F**

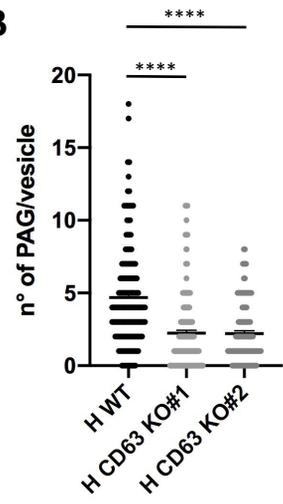


# Supplementary Figure 2

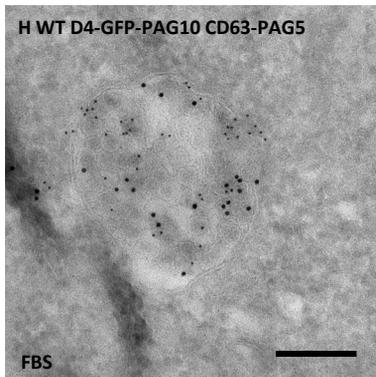
## A



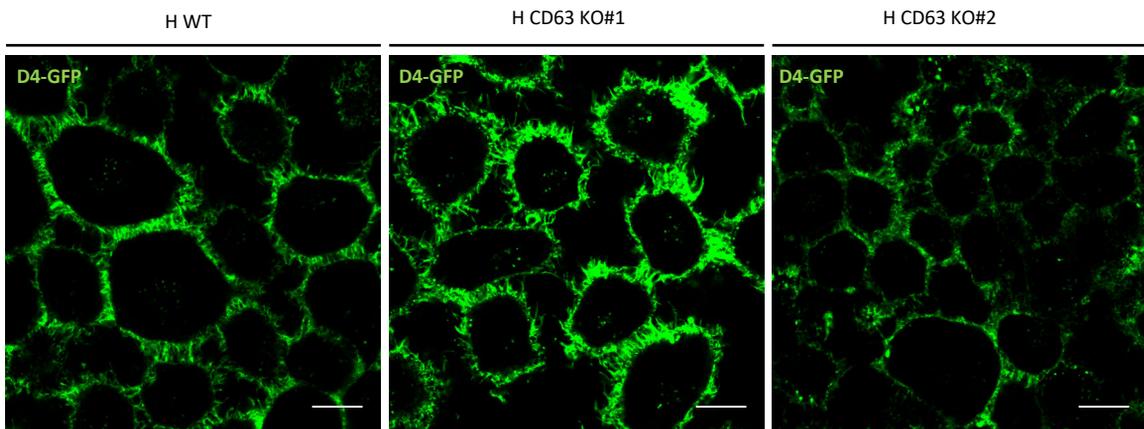
## B



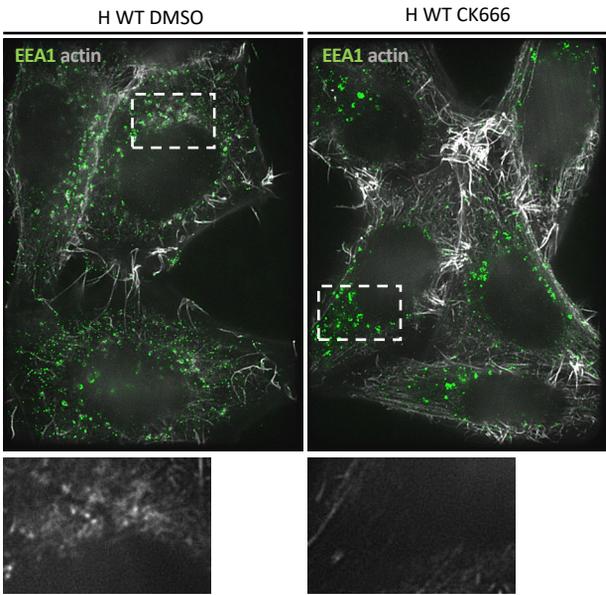
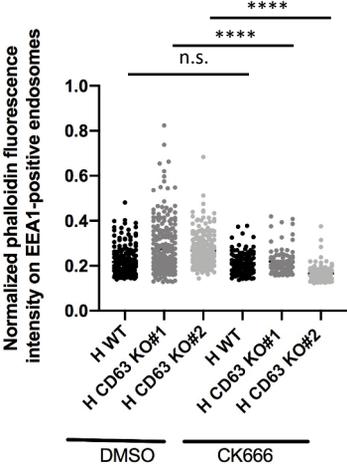
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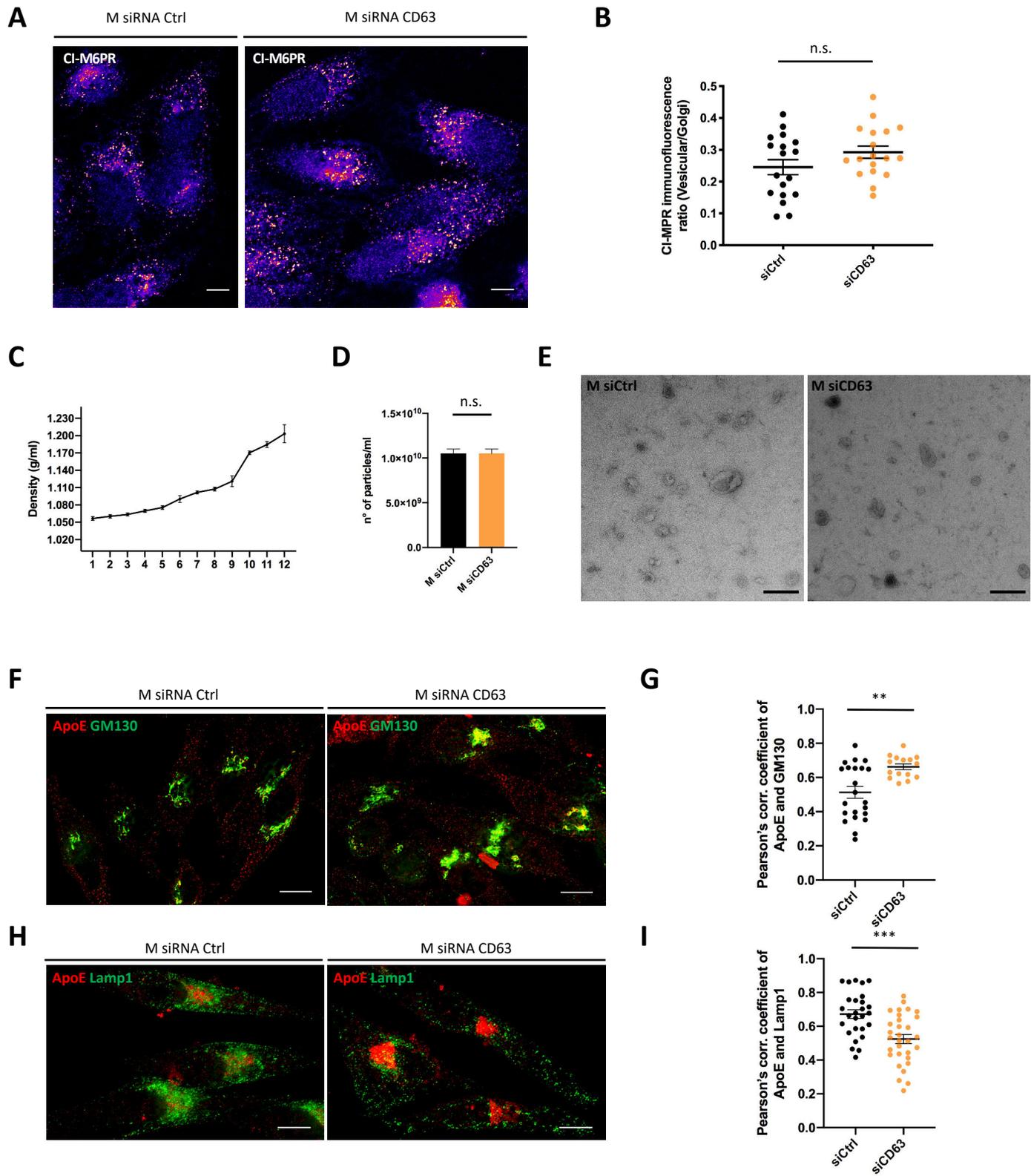
## D



**A** **B**



# Supplementary Figure 4



## **Results, section 3**

Preliminary results: the retromer complex regulates the retrograde transport of  
ApoE



## **Preliminary results: THE RETROMER COMPLEX REGULATES RETROGRADE TRANSPORT OF APOE**

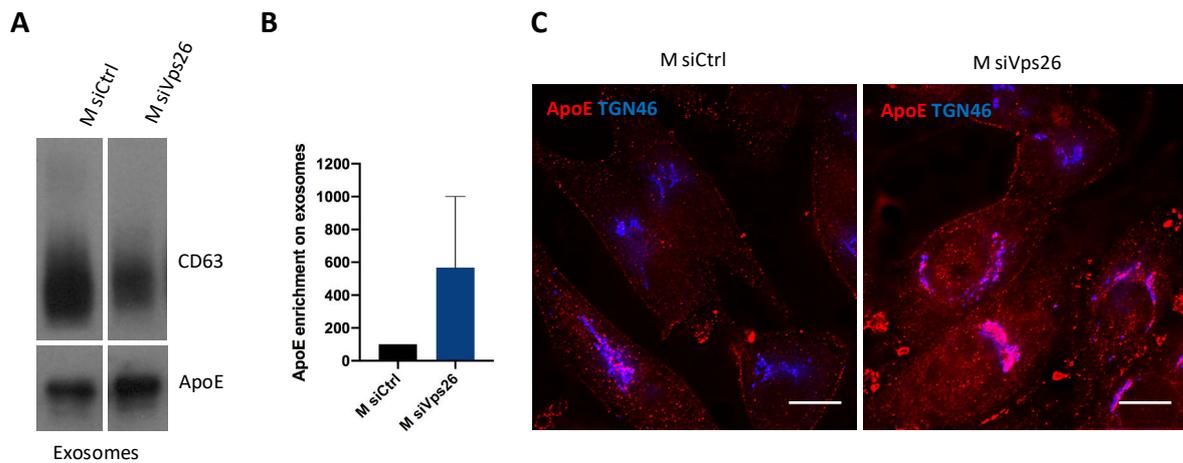
### **Background**

Our current data show that, upon depletion of CD63, cholesterol and ApoE are accumulated in the Golgi apparatus, suggesting that these cargoes exist from endosomes and undergo retrograde transport (**Results, section 2**). This led us to investigate the role of retrograde transport machineries in the egress of ApoE and cholesterol from endosomes. Among the reported machineries we are currently investigating the retromer complex. The retromer complex consists of two subcomplexes: the cargo selective complex, composed by the trimer Vps26/Vps29/Vps35 and a membrane bound dimer composed by the sorting nexins SNX1/2 and SNX 5/6 (Klinger et al., 2015). While the retromer complex participates in cargo recognition and promotes membrane bending to initiate tubule formation, tubule elongation and fission to generate a transport carrier requires actin polymerization. Indeed, the retromer complex has been shown to interact with the WASH complex (Harbour et al., 2012), which in turn regulate the retrograde transport of retromer dependent cargoes such as CI-M6PR (MacDonald et al., 2018). Supporting this hypothesis, we also observed accumulation of endosomal actin patches upon depletion of CD63 (**Results, section 2**). Hence, we hypothesize that upon depletion of CD63, the recruitment of the retromer complex on endosomal membrane is stimulated in order to promote recycling of ApoE and cholesterol, avoiding their endosomal accumulation.

### **Preliminary results**

#### ***1. Depletion of the retromer complex impairs ApoE exosomal secretion.***

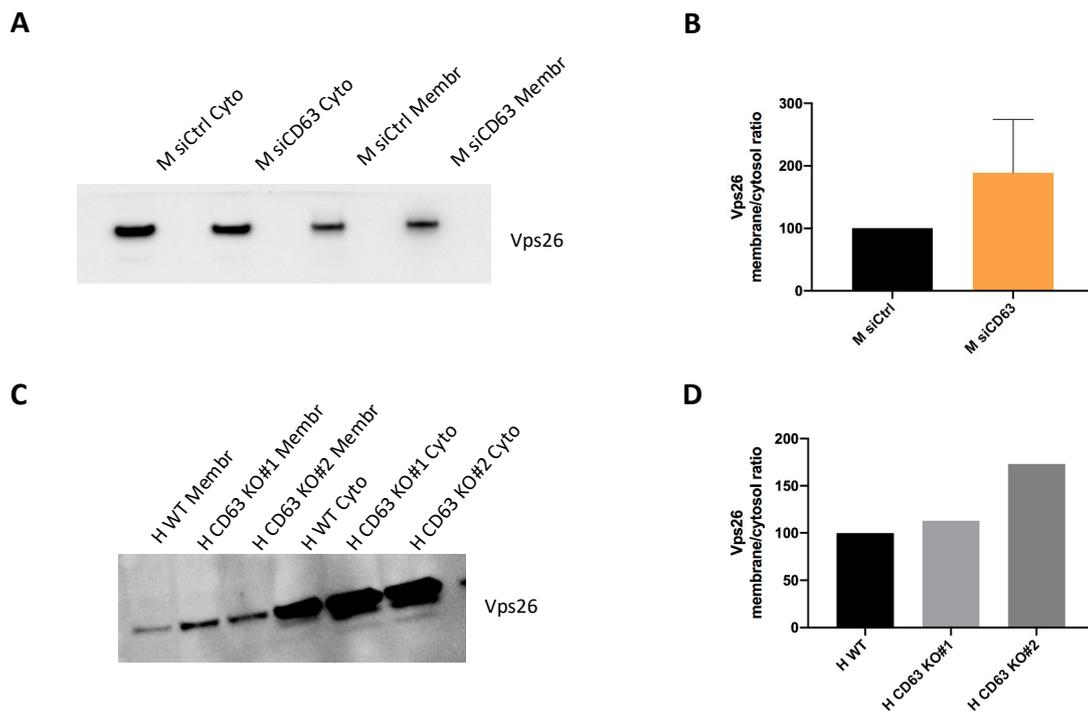
In order to investigate the involvement of the retromer complex in the trafficking of ApoE, MNT-1 cells were treated with Ctrl siRNA or Vps26 siRNA and grown in LPDS supplemented media. EVs from the same number of Ctrl or Vps26 siRNA treated MNT-1 cells were isolated by differential ultracentrifugation and the 100,000 g pellet was recovered and analyzed by WB. Our preliminary results showed that upon depletion of Vps26, exosomal secretion of ApoE is increased (Fig. 1A and 1B). Moreover, we observed an increased endosomal localization of ApoE upon depletion of Vps26 (Fig. 1C). All together these data suggested that the retromer complex was implicated in the retrograde transport of ApoE from endosomes to the Golgi apparatus.



**Figure 1. A.** Western blot analysis of exosomal 100K g pellet from Ctrl or Vps26 siRNA treated MNT-1 cells grown in LPDS. **B.** Quantification of exosomal ApoE content shown in A (n= 2 independent experiments). **C.** IFM of Ctrl or Vps26 siRNA treated MNT-1 cells grown in LPDS and costained for endogenous ApoE and TGN46.

## **2. Endosomal recruitment of the retromer complex is impaired upon CD63 depletion.**

Our data showed that, in absence of CD63, MVEs presented a higher number of tubules/budded structures in their close proximity, suggesting that the formation of tubular/vesicular transport carriers was promoted (**Results, section 2**). Hence, we hypothesized that these tubules may be induced by the endosomal recruitment of the retromer complex. Indeed, preliminary experiments showed that, in both MNT1 and HeLa cells, membrane recruitment of the retromer subunit Vps26 was promoted upon depletion of CD63 (Fig. 2A-D).



**Figure 2. A.** Cytosol-membrane fractions of Ctrl or CD63 siRNA treated MNT-1 cells were immunoblotted using antibodies against Vps26. **B.** Quantification of Vps26 intensity expressed as membrane/ cytosol ratio. **C.** Cytosol-membrane fractions of WT or CD63 KO HeLa cells were immunoblotted using antibodies against Vps26. **D.** Quantification of Vps26 intensity expressed as membrane/cytosol ratio.

## Conclusions and perspectives

Altogether, our preliminary results suggest that the retrograde transport of ApoE in MNT-1 cells is mediated by the retromer complex and that this pathway may be promoted upon depletion of CD63. These observations illustrate how impairment of a retrograde transport pathway would in turn affect exosome composition, as previously reported for the same retromer complex (Sullivan et al., 2011). Nevertheless, it remains to be determined if retromer is involved in the retrograde transport of cholesterol, therefore cholesterol staining will be performed upon retromer depletion in WT and CD63 KO cells. Finally, it remains to be determined how the retromer complex would be recruited to endosomes. Given that a previous study of the host laboratory showed an accumulation of HRS on endosomes upon depletion of CD63 (van Niel et al., 2011), we are currently testing this hypothesis.

## **Additional methods**

### **Membrane cytosol fractionation**

Cells were scraped in cold PBS and centrifuged at 900 rpm for 5 min at 4°C. The pellet was homogenized in homogenization buffer (0.25 M sucrose, 20mM Tris, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, protease inhibitors, pH 7.4) for 10 min at 4°C and cells were lysed using a 27G needle. After centrifugation at 13,000 rpm for 10 min at 4°C, the post nuclear supernatant was collected and centrifuged at 80,000 rpm (rotor TLA 120.2, Beckman Coulter) for 1h at 4°C. The supernatant (cytosol fraction) was collected and the pellet (membrane fraction) was resuspended in equal volume of homogenization buffer. Western Blot were performed as described previously in this manuscript.

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## **Results, section 4**

In vitro interaction of melanoma derived extracellular vesicles with collagen



# IN VITRO INTERACTION OF MELANOMA DERIVED EXTRACELLULAR VESICLES WITH COLLAGEN

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## Abstract

Melanoma derived EVs have been shown to contribute to melanoma progression, by modulating angiogenesis, immune regulation, or by modifying the tumor microenvironment and contributing to the formation of a pre-metastatic niche. Moreover, tumor derived EVs has been proposed to interact and remodel extracellular matrix (ECM) providing a substrate favoring cell adhesion and motility, hence promoting persistent tumor cell migration. Nevertheless, the capacity of EVs to physically interact with ECM components is still questionable. In this study we show that subpopulations of melanoma derived EVs differently physically interact with collagen fibers, the most abundant fibrous protein in ECM. Finally, preliminary data presented in “**Results, section 5**”, suggest that such interaction would modulate collagen fibrillogenesis and melanoma cell migration, supporting the idea that the physical interaction of EVs with the ECM would impact ECM remodeling and tumor cell migration through the tumor microenvironment.

## Introduction

Melanoma is the deadliest skin cancer and among the most aggressive solid tumors, mostly due to its high capacity of metastasizing in distant organs and its resistance to therapies (Paluncic et al., 2016). Metastatic progression of melanoma is a multistep process that includes dysregulation of growth/apoptotic pathways, epithelial to mesenchymal transition, cell migration, loss of keratinocyte control, immune system escape, basal membrane and stroma degradation, formation of metastatic niche and angiogenesis (Bertolotto, 2013).

Melanoma derived EVs have been shown to contribute to several of these steps, including angiogenesis, immune regulation, modification of the tumor microenvironment and formation of a pre-metastatic niche (Hood, 2019). However, the precise role of EVs in these processes remains to be determined.

The tumor microenvironment comprises various cell types, such as fibroblasts and immune cells, and non-cellular components, including soluble factors and the extracellular matrix (ECM) (Wu and Dai, 2017). ECM is a macromolecular network composed of collagens, proteoglycans/glycosaminoglycans, elastin, fibronectin, laminins and other glycoproteins, which not only provide a physical scaffold in which cells are embedded but also contribute to cell differentiation, migration and homeostasis (Theocharis et al., 2016). ECM remodeling is mediated

by a variety of enzymes, including metalloproteases, and occurs in both physiological and pathological conditions. In the context of cancer progression, ECM remodeling contributes to both cell transformation and migration.

Tumor-derived EVs seem to participate to ECM remodeling. Several studies have shown the presence of active proteases (Shimoda and Khokha, 2013) in EVs. Hence, by remaining in the extracellular space, EVs could directly degrade ECM (Giusti et al., 2008; Hakulinen et al., 2008; Lo Cicero et al., 2012). In addition, integrins, the most characterized receptors of ECM components, have been often identified in EVs from different tumor types and they contribute to organotropic metastasis (Hoshino et al., 2015) and tumor growth (DeRita et al., 2019). Finally, ECM components are also associated to EVs. In particular, fibronectin containing exosomes, by association with ECM, would provide a substrate favoring cell adhesion and motility, hence promoting persistent cell migration (Sung et al., 2015).

Nevertheless, very few reports have investigated the capacity of EVs to physically interact with ECM components. Hence, in this study we aimed to determine if melanoma-derived EVs were able to interact with collagen, the most abundant fibrous protein in ECM (Theocharis et al., 2016).

## Results

### Melanoma cells secrete subpopulations of EVs containing ECM components

In order to characterize EVs released by melanoma cells, we choose three melanoma cell lines with different metastatic potential: A375, MNT-1 and WM1716. MNT-1 are pigmented melanoma cells, not tumorigenic when injected in mice (Lazar et al., 2015). A375 and WM1716 are non-pigmented, metastatic, melanoma cell lines. EVs were isolated by differential ultracentrifugation. 100,000 g pellets were recovered and analyzed by Western Blot together with their respective cell lysates (Fig. 1A). As previously shown, MNT1 cells express ApoE, an inhibitor of melanoma progression (Pencheva et al., 2012), and secrete ApoE through exosomes (van Niel et al., 2015); (Results, section 2)). On the contrary A375 and WM1716 cells do not express ApoE, hence exosomes released by these cell lines are negative for ApoE. Interestingly, all the analyzed cell lines express and secrete fibronectin which can be co-recovered with EVs. The presence of fibronectin on EVs may mediate interaction with collagen and in turn promote cancer cell migration (Sung et al., 2015). To further characterize EVs, 100,000 g pellets were loaded onto a bottom-up density gradient (Fig. 1B). 12 fractions were recovered, their density was measured, (Fig.1B) and each fraction was analyzed by WB. As previously shown (**Results, section 2**), MNT1 cells secrete two subpopulations of EVs, corresponding to fraction 6/7 (density of 1,09-1,10 mg/ml) and to fraction 9/10 (density of 1,12 mg/ml). Similarly, A375 derived EVs distribute in different fractions between 1,09 mg/ml and 1,13 mg/ml. Interestingly fibronectin was highly enriched in fraction 9 (and 10 for MNT1) in both MNT1 and A375. Contrarily, only fraction 6 (density of 1,08 mg/ml) contained EVs in the case of WM1716, with this fraction being also enriched of fibronectin.

### **Melanoma derived EVs interact with collagen**

In order to test the capacity of melanoma EVs to interact with ECM, we choose collagen type I, a fibrillar collagen widely spread in the body and main component of the dermis, the first tissue that is invaded by melanoma cells (Theocharis et al., 2016). In order to visualize the interaction of EVs with collagen, EVs were incubated with a thin layer of collagen I, obtained by direct gelation on glass coverslips. The samples were then processed for conventional electron microscopy. As expected, we observed fibrils of collagen with CD63 or CD9 positive EVs that were physically associated to these fibrils (Fig. 2A). To obtain a quantitative measurement of EV-collagen interaction, we performed a pull-down assay of collagen fibrils. 100,000 g pellets of EVs isolated from A375 or MNT1 cells were incubated with polymerized collagen. Collagen fibrils were then pulled down by a low speed centrifugation that allowed to pull down collagen associated EVs, while unbound EVs remained in solution. WB analysis revealed the presence of A375 derived EVs bound to collagen (Fig. 2B). In addition, we observed that the fraction (expressed as % of EV input) of CD9 positive EVs associated to collagen, was higher compared to the fraction of collagen associated CD63 positive EVs (Fig. 2B). Similarly, MNT1 derived EVs were found in the pull down, but no differences were observed between CD63 and CD9 positive EVs (Fig. 2C). Surprisingly, ApoE positive EVs were not found in the pull down, suggesting that ApoE containing EVs do not interact with collagen (Fig. 2C). As control, a pull-down assay was performed with collagen fibrils incubated with PBS instead of exosomes (Fig. 2A and 2B) and, as expected, no signal for CD63 or CD9 was detected. Moreover, fibronectin positive EVs were found in the pull down, suggesting that fibronectin containing EVs would also interact with collagen. Nevertheless, fibronectin was also found when the assay was performed with collagen fibrils incubated with PBS, hence making any observation regarding fibronectin unconceivable (Fig. S1A).

Given the existence of different EV subpopulations in the case of A375 and MNT1 cells, pull down assay was performed using the fractions recovered upon density gradient.

Both fraction 6/7 and fraction 9 of A375 EVs were found to interact with collagen (Fig. 2D). In both cases a higher amount of CD9 positive EVs were pulled down, compared to CD63 positive EVs (Fig. 2D). When MNT1 EVs were analyzed, we observed that fractions 6/7 interact with collagen (Fig. 2E). On the contrary, the ApoE positive fraction 9 was not pulled down with collagen. This was particularly evident for CD63, while a very low amount of CD9 positive EVs was still found (around 2% of the input) (Fig. 2E). Finally pull down was performed with the only EV containing fraction from WM1716 cells (Fig. 2F). In this case, association of CD63 or CD9 positive EVs with collagen was also observed, again with a majority of CD9 positive vesicles being pulled down (Fig. 2F).

## Discussion

In this study we characterized small EVs secreted by three different melanoma cell lines and we tested their capacity to interact with collagen.

Despite the capacity of EVs to remodel or degrade ECM has been shown in different studies (Giusti et al., 2008; Hakulinen et al., 2008; Lo Cicero et al., 2012; Mu et al., 2013), these studies did not illustrate the physical interaction of EVs with collagen. To our knowledge, the only study addressing this question used FACS to study the interaction of rat pancreatic cancer derived EVs with single ECM components, including collagen I (Mu et al., 2013). Hence, our study reports for the first time the capacity of melanoma derived EVs of physically interacting with collagen I, as observed using electron microscopy and pull down assay.

Different studies have analyzed the protein composition of melanoma EVs, which differs from normal melanocytes and among different cell lines (Lazar et al., 2015; Xiao et al., 2012). Moreover, similarly to other studies, we found that at least some melanoma cell lines secrete multiple subpopulations of EVs that can have different roles (Willms et al., 2016).

Our pull down assays suggested that CD9 positive vesicles bind more efficiently to collagen than CD63 positive EVs for the three cell lines. Kowal et al. identified a subpopulation of CD9 positive EVs that do not contain CD63 and contain specific proteins not found in CD63 positive EVs (Kowal et al., 2016). Moreover, considering the major plasma membrane localization of CD9 and the major endosomal localization of CD63 in most cell types, it is possible that CD63 and CD9 positive subpopulations have different origin. This would explain their different composition and hence, their different functions.

Among the proteins that may influence the interaction of melanoma derived EVs with collagen, we found fibronectin on EVs of the three cell lines, confirming proteomic studies performed at least on MNT-1 cells by us (unpublished data, not shown) and others (Lazar et al., 2015). Fibronectin is a component of the ECM which also binds collagen, preferentially denaturated (Dessau et al., 1978). In addition, binding of fibronectin to collagen seem to promote destabilization of collagen fibers, suggesting a role for fibronectin in collagen remodeling (Erat et al., 2009). However, fibronectin was only present in the fraction 9-10 of MNT-1 derived EVs that did not interact with collagen. Hence, the presence of fibronectin on exosomes does not seem to always correlate to the capacity of EVs to interact with collagen. These results would also suggest that fibronectin on EVs is dispensable for EV-collagen interaction and consequent promotion of cell migration as previously proposed (Sung et al., 2015).

In this study we selected the non-tumorigenic pigmented melanoma cell line MNT-1 and two amelanotic metastatic cell lines, A375 and WM1716. Contrarily to MNT-1, A375 and WM1716 do not express ApoE. Our previous study illustrate the role of ApoE in the biogenesis of melanosomes and pigmentation of melanocytes and melanoma cells (van Niel et al., 2015). Given that both A375 and WM1716 are amelanotic cell lines, hence not pigmented, it is likely that the expression of genes involved in pigmentation is decreased. Indeed, A375 (Vachtenheim et al., 2001) and WM1716 (Golan et al., 2015) express low levels of the transcription factor MITF, a master regulator of pigmentation, of which ApoE is a target gene (Hoek et al., 2008). Moreover, ApoE has been described as an inhibitor of melanoma metastasis with ApoE expression and secretion being

negatively correlated with metastatic potential (Pencheva et al., 2012). Hence, being A375 and WM1716 tumorigenic and metastatic cell lines, our study confirmed this correlation.

We observed that ApoE containing EVs do not seem to interact with collagen. Our previous work described ApoE as a CD63 dependent cargo suggesting that ApoE is present on CD63 positive EVs while it may not be present on CD9 positive EVs (**Results, section 1 and section 2**). The observation that in fraction 9 of MNT1 CD63 and also ApoE are not pulled down with collagen would further support this model. This results also suggest that ApoE could negatively regulate the interaction of EVs with collagen but the reason remains to be determined. Of note, the lipidation state of ApoE may also influence its capacity of interacting with the ECM (Burgess et al., 1998). Hence, ApoE association to exosomes, a lipid particle, might influence its capacity of interacting with the ECM. Yet, it cannot be excluded that ApoE-EVs would interact with other components of the ECM such as heparan sulphate proteoglycans (HSPGs), which are known receptors of ApoE. Indeed, ApoE has been shown to bind hepatocyte derived ECM, in particular their HSPGs (Burgess et al., 1998). Moreover, HSPGs are also expressed on the cell surface, where they bound ApoE and facilitate ApoE internalization together with the receptor LRP1 (Ji et al., 1993). Being ApoE/LRP1 interaction important for melanoma cell migration (Pencheva et al., 2012), we cannot exclude that, in vivo, at least a pool of ApoE-exosomes remains close to the cell surface of melanoma cells to interact with LRP1, mediating a signaling cascade that results in the inhibition of melanoma cell migration.

Finally, among the known collagen receptors, integrins have been largely found in EVs. Despite we were not able to detect integrins by WB analysis, Integrin  $\beta$ 1 has been identified by mass spectrometry, at least in MNT-1 cells, by us (unpublished data, not shown) and by others (Lazar et al., 2015). Therefore, integrins remain good candidates for collagen- EV interaction.

In conclusion our study showed that melanoma cell lines secrete subpopulations of EVs with different composition. We also reported for the first the time that melanoma derived EVs physically interact with collagen and that this property is dependent on EV protein composition. Such process may in turn contribute to melanoma metastatic progression by promoting ECM remodeling and/or by favoring the migration of melanoma cells.

## Materials and methods

### Cell culture, cell lysates and Western Blot

A375 cells were maintained in DMEM supplemented with 10% FBS and penicillin-streptomycin (Gibco). MNT-1 cells were maintained in DMEM supplemented with 20% FBS, 10% AIM-V medium, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin. WM1716 were maintained in RPMI supplemented with 10% FBS and penicillin-streptomycin. For exosome recovery cells were maintained in the same medium, depleted for bovine-EVs (obtained by overnight ultracentrifugation at 100,000 g).

Cells were lysed directly in lysis buffer (20 mM Tris, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, pH 7.2) with protease inhibitor cocktail (Roche). Western Blot analysis was performed as described in **Results, section 1**. Signal intensities were quantified with Image J Fiji software.

### EV isolation and Optiprep density gradient

EVs were prepared from conditioned media incubated for 48 hr on sub-confluent cells. EVs were isolated by ultracentrifugation, as described in **Results, section 2**, and purified using OptiPrep density gradient centrifugation as previously described in **Results, section 2**, when indicated. The resulting pellets were resuspended in PBS (pH 7).

### Antibodies and reagents

Antibodies and their sources were as follows: anti-CD63 (ab23792, 1:200 dilution for WB and EM), anti-CD9 was a kind gift of Eric Rubinstein (Inserm, U935, Villejuif, France; dilution 1:500 for WB and 1:200 for EM), anti-ApoE (ab52607, dilution 1:500 for WB), anti-fibronectin (Sigma, F3648, dilution 1:1000 for WB), anti-collagen (ab292, dilution 1:1000). Horseradish peroxidase (HRP)-conjugated goat polyclonal antibodies to rabbit IgG and to mouse IgG were from Abcam and were used at 1:10000 dilution. Protein A conjugated to 5 or 10 nm gold particles (PAG; 1:50; Cell Microscopy Center, Utrecht University Hospital, Utrecht, Netherlands). Rat tail Collagen I was from Corning.

### Electron microscopy

For conventional electron microscopy, Collagen I mix was prepared by adding 10X PBS, 1M HEPES and H<sub>2</sub>O and the pH was neutralized by adding 1M NaOH. Gelation was conducted on coverslips for 10 minutes at RT. Exosomes were added on top of the collagen gel and incubated for 20 minutes at RT. Coverslips were fixed with a mixture of 2% PFA and 0.2% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, and processed for immunogold labeling using anti-CD63 or anti-CD9 antibody and PAG 10nm. Coverslips were then fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, processed for Epon embedding and ultrathin sections and contrasted with uranyl acetate and lead citrate as described in **Results, section 2**. The samples were analyzed with an 80 kV transmission electron microscope (Tecnai Spirit G2; Thermo Fischer, Eindhoven, The Netherlands) equipped with a 4k CCD camera (Quemesa, EMSIS, Münster, Germany).

**Pull-down assay**

Collagen I mix was prepared by adding 10X PBS, 1M HEPES and H<sub>2</sub>O and the pH was neutralized by adding 1M NaOH (final concentration of collagen 6,5 mg/ml). Gelation of collagen was conducted in « low retention » tubes for 10 minutes at RT and stopped by adding cold PBS and keeping samples in ice. Collagen gels were then sonicated on ice 3 times for 10s, with 10s breaks in between, at 50% amplitude. EVs, isolated as described above, were added to the collagen gels and the samples were incubated for 2h 30min at RT. One volume of PBS was added and samples were sonicated on ice 12 times for 10s, with 10s breaks in between, at 50% amplitude. Collagen fibrils were pulled down by centrifugation at 2,150 g for 1h at 4°C. The supernatant (unbound fraction) was recovered and the volume was reduced using Microcon Centrifugal Filter Devices (Millipore) according to manufacturer's instructions. Pellets (pull down) were washed with cold PBS, resuspended in PBS and processed for WB as described above. The same volume of EVs used for the pull down, was analyzed by WB (input). As control, pull down assay was performed with collagen incubated with PBS instead of EVs.

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## Figure legends

### **Figure 1. Characterization of EVs released by different melanoma cell lines.**

**A.** Western Blot analysis of cell lysates and 100K g exosomal pellet from the melanoma cell lines A375, MNT-1 and WM1716. **B.** Density values of the fractions recovered from a bottom-up Optiprep density gradient of 100K g exosomal pellet from A375, MNT-1 and WM1716 cells (mean  $\pm$  SEM, 3 independent experiments). **C.** Western-blot analysis of a bottom-up Optiprep density gradient of 100K g exosomal pellet from MNT-1 cells. **D.** Western-blot analysis of a bottom-up Optiprep density gradient of 100K g exosomal pellet from A375 cells. **E.** Western-blot analysis of a bottom-up Optiprep density gradient of 100K g exosomal pellet from WM1716 cells.

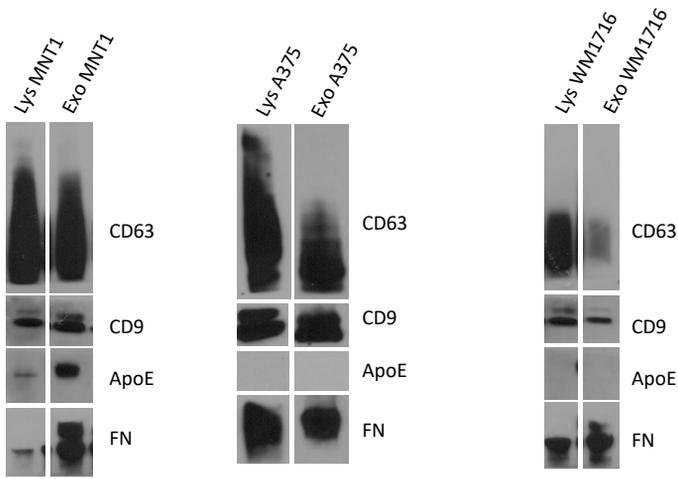
### **Figure 2. Melanoma derived EVs physically interact with collagen.**

**A.** A375 exosomes were incubated with collagen, pre-stained with CD63 or CD9 (PAG-10) and analyzed by conventional EM. Arrows indicate stained EVs associated with collagen. **B-F.** WB analysis of a pull down of collagen fibrils incubated with exosomes. Quantifications of the percentage of CD63 or CD9 positive vesicles that were pulled down with collagen are shown (mean  $\pm$  SEM, 3 independent experiments).

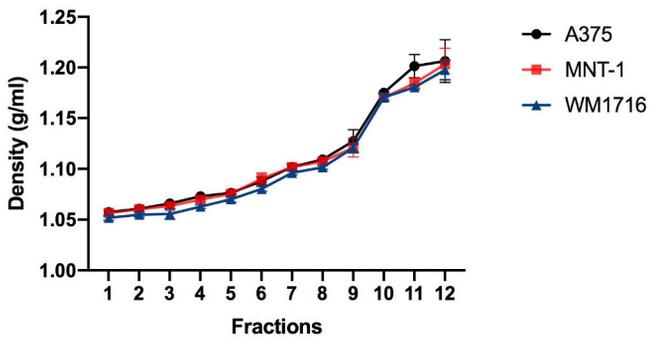
**Supplementary figure 1. A.** WB analysis of a pull down of collagen fibrils incubated with exosomes. A representative blot for fibronectin is shown.

Figure 1

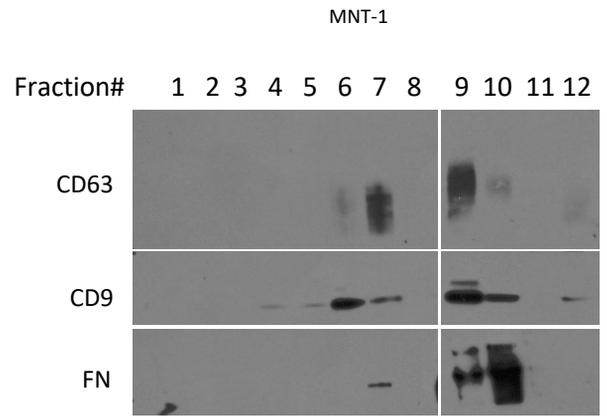
**A**



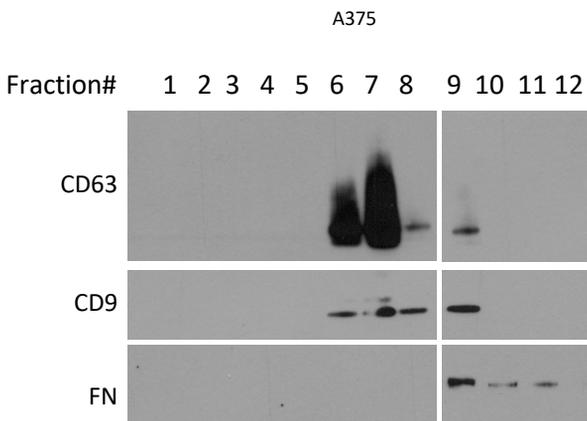
**B**



**C**



**D**



**E**

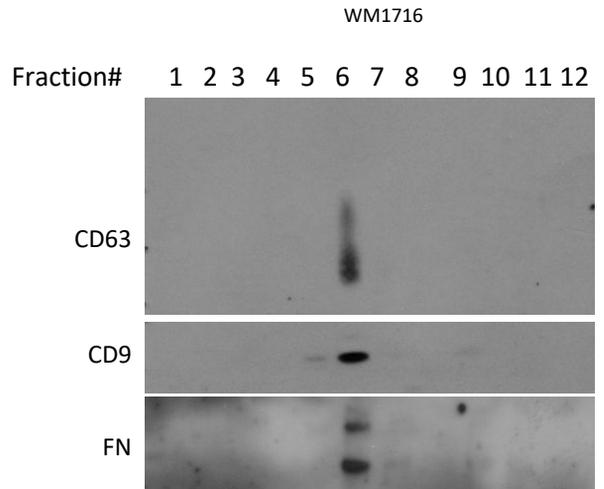
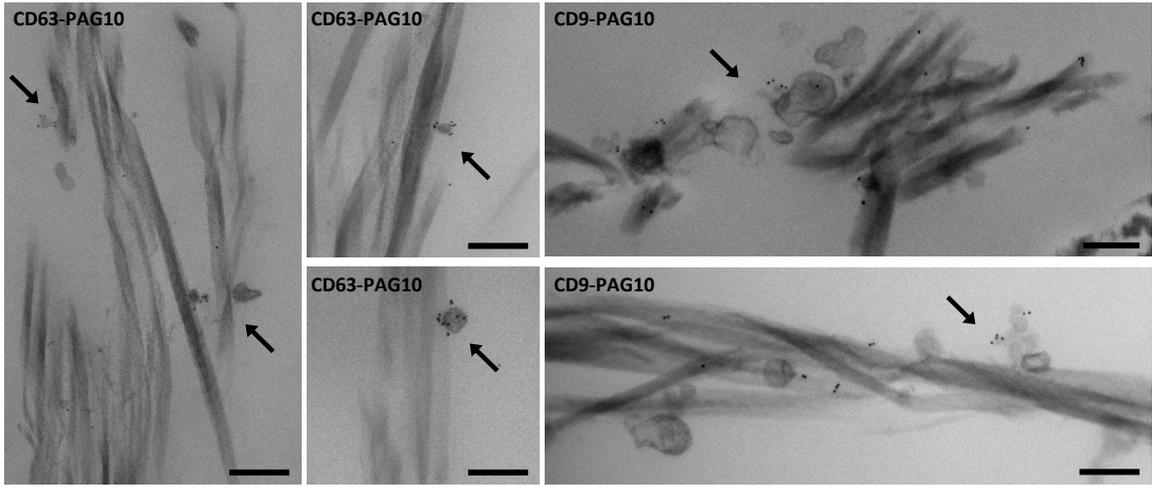
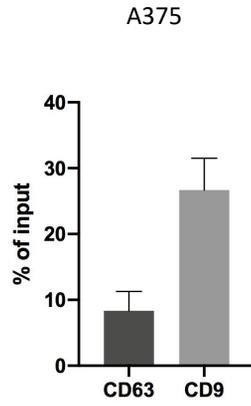
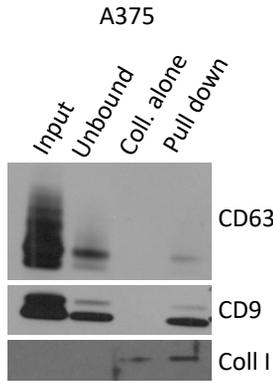


Figure 2

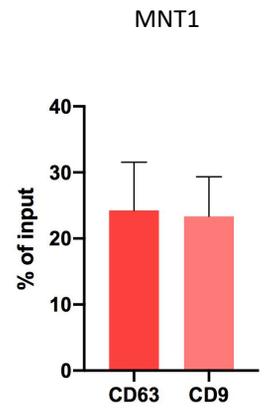
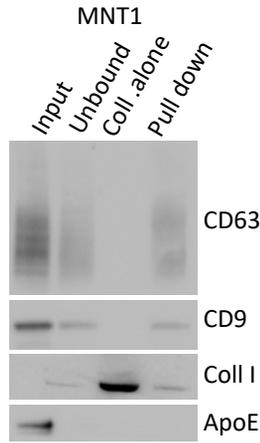
**A**



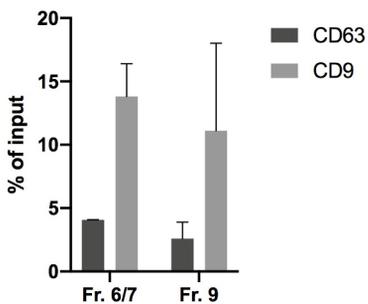
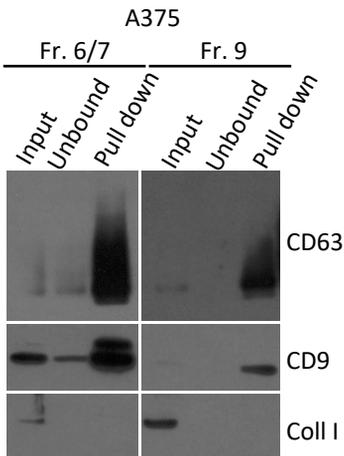
**B**



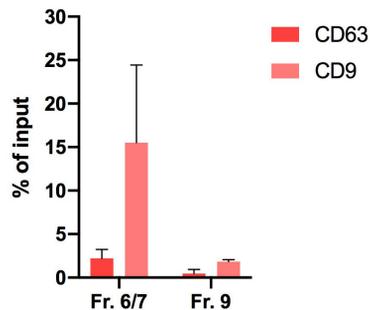
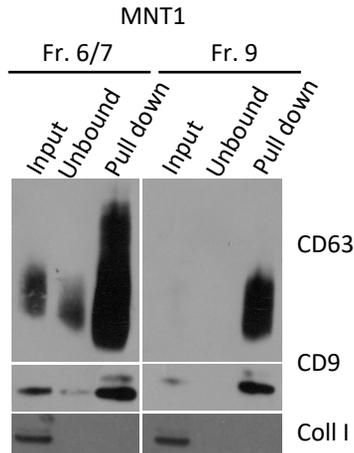
**C**



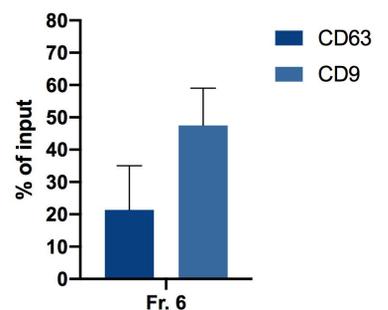
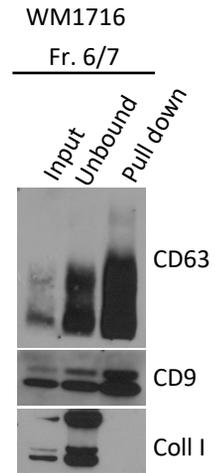
**D**



**E**

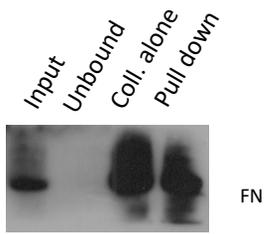


**F**



# Supplementary Figure 1

## A



## **Results, section 5**

Preliminary results: EV-collagen interaction promotes ECM remodeling and influences melanoma cell migration



## Preliminary results: EV-COLLAGEN INTERACTION PROMOTES ECM REMODELING AND INFLUENCES MELANOMA CELL MIGRATION.

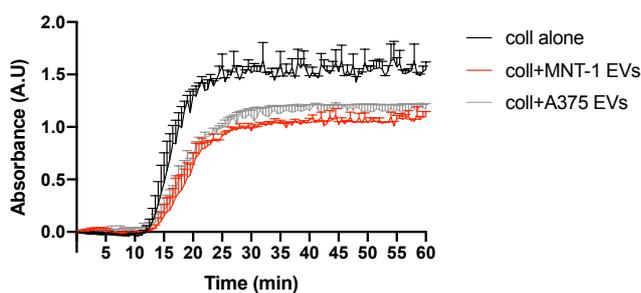
### Background

Considering the capacity of melanoma derived EVs to interact with collagen (**Results, section 4**), we investigated if this interaction could have functional implications in ECM remodeling or in modulating the migration of melanoma cells on 3D collagen.

### Preliminary results

#### 1. EVs may impair collagen fibrillogenesis

In vitro collagen fibrillogenesis was monitored by measuring the turbidity of a collagen solution (Zhu and Kaufman, 2014) as it assembled into fibers upon warming monomeric collagen solutions from 4 °C to 37 °C, in presence or not of EVs derived from MNT-1 cells or A375 cells. The increase in turbidity (growth phase) is indicative of collagen fibril formation until a plateau phase is reached. The plateau value depends on the amount of collagen fibers present in the solution, on the diameter of the fibers and on their cross-linking. We observed a slight delay in the starting of the growth phase in presence of EVs from either MNT-1 cells or A375 cells. Moreover, the final absorbance was higher in the case of collagen alone (**Fig. 1**). These data suggest that EVs may slow down collagen fibrillation or affect the structure of the collagen fibrils or their cross-linking. Nevertheless, we cannot discriminate if exosomes would impair the assembly of collagen fibrils or if exosomes would promote the disassembly of collagen fibrils, for instance by promoting collagen degradation or remodeling.



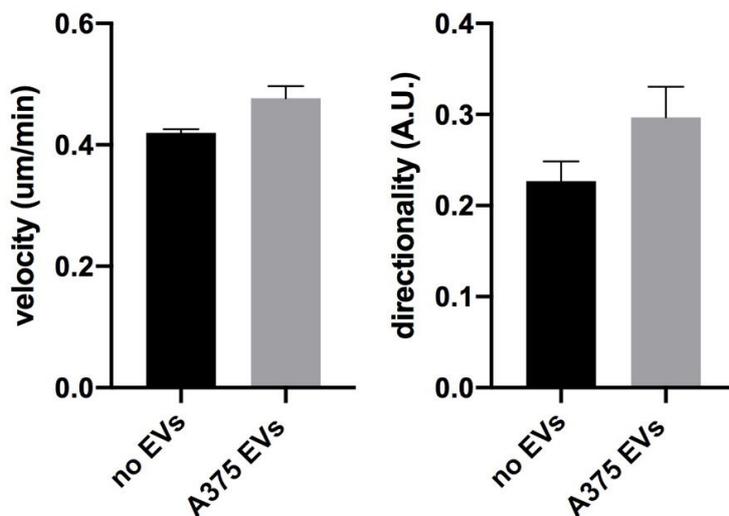
#### Figure 1. Effect of exosomes on collagen fibril formation.

Collagen fibril formation was recorded by measuring the turbidity of 1 mg/ml collagen solutions at 37°C. Individual curves indicate collagen alone (black) or in presence of MNT-1 exosomes (red) or A375 exosomes (gray). Results are shown as mean+ SEM from 2 independent experiments.

#### 2. EVs may promote the directional migration of melanoma cells in a 3D collagen matrix

In order to establish if the interaction of exosomes with collagen may promote the migration of melanoma cells in the extracellular matrix by favoring the adhesion of cancer cells to the ECM, as previously suggested for fibrosarcoma cells (Sung et al., 2015), we performed a migration assay of melanoma cells in a 3D collagen matrix coated or not with EVs. In order to do this, we prepare

collagen fibrils coated with EVs using the same protocol used for the pull down assay (as described in “**Results, section 4**”). EV-coated or non-coated fibrils were then mixed with a new collagen solution and with cells to create a 3D collagen network. Migrating cells were then imaged overnight and manually tracked. Unfortunately, both MNT-1 and A375 cells were not able to migrate, and in some cases, they did not survive, in the 3D collagen network. Only WM1716 cells were able to migrate and their position could be tracked over time. Our preliminary results suggest that the presence of A375 derived EVs in the collagen matrix would increase the speed of WM1716 cells and the directionality of their migration (**Fig.2**). Of note, the assay was also performed in presence of collagen fibrils coated with EVs from MNT-1 cells or WM1716 cells, but the results were not conclusive. Overall, our preliminary data suggest that EVs may promote the migration of melanoma cells and promote their persistent migration. Nevertheless, optimization of the assay is still required. First, our pull down assay (**Results, section 4**) showed that on average only the 15% of EVs were pulled down. Hence, it might be that the amount of EVs present in the 3D collagen matrix was not sufficient to significantly change the migratory behavior of melanoma cells. Moreover, it is likely that the coating of EVs on the collagen network was not uniform, therefore cells that were in close proximity of EVs could migrate differently compared to cells that were far from EV coated collagen fibrils.



**Figure 2. Exosomes may influence the migration of melanoma cells in 3D collagen matrix.**

WM1716 cells migrating in a 3D collagen matrix coated or not with A375 derived EVs were manually tracked. Average velocity ( $\mu\text{m}/\text{min}$ ) and average directionality are shown. Data are shown as mean+SEM from 3 independent experiments.

## Conclusions and perspectives

In conclusion, our preliminary data suggest that, by interacting with collagen, EVs released by melanoma cells could influence collagen structure and the migration of melanoma cells in a 3D extracellular matrix. Indeed, EVs have been previously shown to remodel ECM by degrading collagen (Giusti et al., 2008; Hakulinen et al., 2008) or by promoting collagen crosslinking (de Jong et al., 2016). Live imaging of collagen fibrillation might help to better understand the structure of the collagen matrix in the presence of EVs. Furthermore, melanoma EVs may promote adhesion of melanoma cells on collagen, hence favoring migration. The initial adhesion and spreading of

melanoma cells on a collagen layer, in presence or not of EVs, could then be tested. Moreover, EVs have been shown to promote cancer cell chemotaxis (Sung and Weaver, 2017). Hence, migration of melanoma cells on collagen containing a gradient of EVs should be tested. Finally, because we showed that ApoE positive EVs do not interact with collagen, the 3D migration assay performed here would have not allowed us to study the possible roles of ApoE-EVs. Hence, alternative methods, such as use of different ECM matrix should be performed.

## **Additional materials and methods**

### **Collagen turbidity measurement**

Collagen I mix was prepared as described above to obtain a final concentration of 1 mg/ml of collagen. Collagen was mixed with PBS or exosomes from MNT-1 or A375 cells. Turbidity of collagen gels forming at 37°C was monitored at 400 nm using a spectrophotometer every 30 seconds for 1 hour. Measurements were averaged across three samples per experiment. Absorbance values of the reference solution (buffer without collagen) or of exosomes alone were subtracted from the sample values.

### **3D migration assay**

For 3D cell migration assays, collagen fibrils coated with exosomes were prepared as described for the pull-down assay (**Results, section 4**). The pellet was mixed with a new collagen mix at a final concentration of 2,2 mg/ml. 50 ul drops of this mix were seeded with 20,000 WM1716 cells and polymerized on glass-bottom wells in a 96-well plate at room temperature for 30 minutes, before immersing the set-up in pre-warmed complete medium. The next day, cells were imaged by phase contrast microscopy every 20 min for 14 hours, acquiring a single optical section and several positions per well. Manual tracking of migrating cells was performed using Image J Fiji software. At least 30 cells per conditions and per experiments from 3 independent experiments were analyzed.

## **Acknowledgments**

This work was performed in collaboration with the laboratory of Guillaume Montagnac (Inserm U1170, Gustave Roussy Institute, Université Paris-Saclay, Villejuif, France). I thank Guillaume Montagnac and Enzo Breteau for their contribution in the conceptualization of experimental procedures, for technical assistance, protocols and insightful scientific discussions.

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# **DISCUSSION**



## DISCUSSION

The aim of my thesis was to elucidate the role of endosomal CD63 in the sorting of cargoes to ILVs and in exosome biogenesis. I showed that CD63 regulates the sorting of cholesterol into ILVs, hence influencing exosome composition. In specialized cell types, such as pigment melanoma cells, such pathway contributes to the sorting of ApoE into ILVs, which regulates early pigmentation. Moreover, secretion of ApoE on exosomes seem to have an impact on the function of exosomes during melanoma progression, in particular on the capacity of melanoma derived exosomes to interact with the extracellular matrix.

In this section I will discuss how this work provides a better understanding of CD63 molecular functions and how this pathway influences exosomes composition, hence exosome functions.

### 1. New insights in the role of CD63

#### 1.1. CD63 regulates multivesicular endosome homeostasis

ILV formation is an essential step during the process of endosome maturation. Among the mechanisms described so far, we focus our study on the role of the tetraspanin CD63. Initial studies on the role of the ESCRT machineries in ILV formation showed that in absence of ESCRT components CD63 was still present in ILVs, suggesting that CD63 may have a role in ESCRT-independent ILV biogenesis [208]. This hypothesis was further supported by the observation that CD63 participates in the biogenesis of small ILVs in HeLa cells when the ESCRT-0 component HRS is depleted [203]. Previous studies from my host laboratory illustrated the role of CD63 in the sorting of the melanosomal protein PMEL into ILVs of Stage I melanosomes, the early endosomal compartment of pigment cells [139]. Nevertheless, any of these studies addressed the consequences of CD63 dependent ILV formation on exosome secretion. We showed here that depletion of CD63 in both HeLa cells and MNT-1 cells does not alter the number of secreted exosomes (**Results, section 2**). One possibility is that in absence of CD63, other biogenesis pathways (e.g. ESCRT dependent pathway) may be overactivated. Yet, as previously observed [203], we did not observe a change in ILV number in HeLa cells while a previous study from the host laboratory observed a decreased number of ILVs in stage I melanosomes of MNT-1 cells [139]. All together, these observations suggest that CD63 alone is not sufficient to generate ILVs. In this respect, our data show that, at least in MNT-1 cells, exosomal release of CD63 and ApoE are not impaired upon modulation of the ESCRT-dependent or the ceramide-dependent mechanism (**Results, section 2**). Therefore, we are currently investigating the involvement of the syntenin-alix pathway given that syntenin is a known direct interactant of CD63 [223] and that syntenin seem responsible of CD63 exosomal release both in vitro and in vivo systems [205], [207]. Yet, CD63 is required for the sorting of specific cargoes to ILVs and future exosomes. Here we could identify not only a protein cargo, ApoE, but also a lipid cargo, cholesterol (**Results, section 1 and 2**). Moreover, we showed that in absence of CD63 both cholesterol and ApoE

are accumulated in the Golgi apparatus (**Results, section 2**), suggesting that CD63 may regulate transport pathways between the endosomes and the Golgi apparatus.

Accumulation of CD63 dependent cargoes in the Golgi apparatus has been already observed in the case of neutrophil elastase in neutrophils [341]. Despite it cannot be excluded that CD63 would regulate the anterograde trafficking of neutrophil elastase, this does not seem to be the case for melanosomal cargoes in MNT-1 cells. Several observations support this hypothesis. First, in absence of CD63 both ApoE and PMEL are still targeted to Stage I melanosomes in MNT-1 cells. Moreover, while lysosomal proteins missing a lysosomal sorting motif, such as Syt VII [331], hijack the CD63 pathway, PMEL contain a dileucine based motif [131], likely recognized by AP-2, mediating its internalization, likely independently of CD63. Hence, we believe that at least in melanocytes, CD63 is not required for the anterograde transport of PMEL and ApoE but that CD63 would encounter these cargoes in the endosomal compartment and maintain the balance between their sorting into ILVs and their retrieval from endosomes.

As described, after proteolytic cleavage, the luminal domain of PMEL is sorted into ILVs in a CD63 and ApoE dependent manner. This process is essential for melanosome biogenesis. We previously observed that upon depletion of CD63 the full length PMEL is sorted for lysosomal degradation [139], suggesting that CD63 regulates the balance between sorting into ILVs, hence melanosome biogenesis, which is ESCRT-independent, and fusion with lysosomes for cargo degradation, which is dependent on the ESCRT complex. A recent study illustrates a similar role for CD63 in determining the fate of nascent insulin granules toward degradation versus secretion [482]. Furthermore, CD63 seem to also bridge exosome secretion with autophagy, as in the case of Epstein-Barr virus infected HEK293 cells [263]. By regulating the sorting of the EBV-derived protein LMP-1 into ILVs, CD63 controls signaling pathways that in turn regulates autophagy. Despite LMP-1 mediated signaling per se influences autophagy in infected cells, CD63 may regulate the interactions between endosomal and autophagic processes also in non-infected cells.

All together these data illustrate that CD63 not only regulates cargo endosomal trafficking but also determines the fate of MVEs by maintaining a balance between secretory pathways (exosomes, LROs) and degradative/recycling pathways (degradation in lysosomes/autophagosomes or retrograde transport). Hence CD63 appears to be a main regulator of endosomal homeostasis and dynamics.

### **1.2. CD63 regulates retrograde transport pathways from endosomes to Golgi**

Supporting the hypothesis that CD63 regulates cargo egress from endosomes, we observed an increase of tubular/vesicular transport carriers emanating from endosomes. Such carriers may be required for the retrograde transport of cargoes to the Golgi apparatus. Indeed, our data show that the CD63 -dependent cargoes ApoE and cholesterol are accumulated in the Golgi apparatus upon CD63 depletion. Although we have not yet fully identified the molecular machinery involved, some hypothesis can be drawn. As described in the introduction, the egress of cholesterol from endosomes can be mediated by non-vesicular

transport or vesicular transport. While we could not observe a change in membrane contact sites between ER and MVB, we could observe occasionally the presence of cholesterol in tubular/vesicular carriers, hence we hypothesized that the transport of cholesterol was mediated by vesicular transport. Among the possible pathways that may mediate the retrograde transport of both cholesterol and ApoE, we are currently investigating the role of the retromer complex. Contrarily we did not investigate other pathways such as Rab9 that is involved in trafficking pathways between endosomes and Golgi [67] and in the egress of cholesterol from endosomes [379], for several reasons. First, Rab9 associates to vesicular carriers while it does not seem to associate with tubular transport carriers [66]. In addition, while Rab9 overexpression is able to rescue endosomal cholesterol accumulation induced by NPC1 mutation, depletion of Rab9 does not seem to affect cholesterol localization, especially in HeLa cells [379]. Nevertheless, Rab9 has been shown to cooperate with the retromer complex and actin in the retrieval of cargoes from endosomes in *Drosophila* [483], hence we cannot exclude that Rab9 might act as an upstream regulator of a retromer mediated pathway.

Alternatively, we had previously shown that CD63 depletion in MNT-1 cells induce accumulation of clathrin and HRS coats on the delimiting membrane of endosomes [139]. Interestingly both clathrin and HRS participates not only to the ESCRT-mediated degradative pathway [29], but also to the recycling of cargoes to the TGN in cooperation with the retromer complex, as illustrated for STxB [484] or CI-M6PR [106]. Moreover, HRS mediates egress of cholesterol from endosomes by an unknown mechanism [376]. In addition, HRS has recently been shown to recruit the actin nucleation factor WASH on endosomal subdomains [106], which is also a known interactant of the retromer complex [105]. As illustrated in “**Results, section 2**”, we observed an accumulation of actin patches around endosomes upon depletion of CD63, suggesting that actin is required for the formation of tubular/vesicular carriers. Considering the well-known cooperation of actin and the retromer complex in this process [485], it seems likely that the observed tubulations can be mediated by the retromer complex.

Our preliminary data show that depletion of the retromer subunit Vps26 in MNT-1 cells induces an increase in ApoE secretion on exosomes and an increase in the endosomal pool of ApoE (**Results, section 3**), suggesting that the retromer complex may be involved in the retrograde transport of ApoE to the Golgi apparatus. This observation also illustrates how impairment of the retrograde transport can affect exosome composition and secretion. In addition, depletion of CD63 seem to increase the membrane recruitment of Vps26 (**Results, section 3**), suggesting that upon depletion of CD63, endosomal recruitment of the retromer complex might be promoted in order to counterbalance the accumulation of cargoes on the delimiting membranes of endosomes. Finally, mass spectrometry analysis of the interactome of SNX-BAR proteins identified CD63 as a possible interactor of SNX5 [78]. Therefore, it is tempting to speculate that, at steady state, CD63 may regulate the balance between cargo sorting into ILVs and recycling through the retromer complex. All together these data would illustrate for the first time the involvement of the retromer complex in the transport of

cholesterol and in the transport of ApoE. Nevertheless, some questions remain open: how is retromer initially recruited and how is cholesterol sorted into the tubular domain? Apart from the known role of PI3P and Rab7 (among others) that can contribute to recruitment and stabilization of the retromer domain, we cannot exclude that a protein cargo with affinity for cholesterol enriched microdomains might participate in the recruitment of the retromer complex. In addition, it has been shown that cholesterol preferentially accumulates in high curvature regions of membranes [486], suggesting that cholesterol would tend to accumulate in nascent buds/tubules.

### **1.3. CD63 regulates sorting of cholesterol into ILVs**

Our study shows that CD63 regulates the sorting of cholesterol into ILVs and future exosomes. Such process could be dependent on the specific organization of CD63 and cholesterol to create tetraspanin enriched microdomains (TEMs). Indeed precipitation of cholesterol, using the detergent digitonin, has shown the interaction of cholesterol with tetraspanins [310] that may stabilize the interaction of tetraspanins with each other. In addition, palmitoylation of tetraspanins seems to participate to their interaction with cholesterol [310] and to stabilize the interaction between distinct tetraspanins and related partners [307], [308]. Alternatively, the determination of the crystal structure of CD81 revealed the presence of an intramembrane cavity within the transmembrane region in which cholesterol can be allocated [222]. Of note, cholesterol- bound and -unbound CD81 have a different tridimensional conformation (**Figure 17**), that can influence the function of CD81, including the interaction of the extracellular loop with other partners. This cholesterol-containing pocket can be present in other tetraspanins including CD63. Determination of the crystal structure of CD63 or use of different detergents to isolate CD63 or CD63 enriched microdomains will definitively help to answer this question. So far, our data show that the trafficking of cholesterol into ILVs is dependent and linked to CD63, but we could not discriminate if cholesterol is only present in the microdomain or more specifically allocated inside a CD63 pocket. But how is cholesterol inserted into the tetraspanin pocket? First, it should be considered that in the case of CD81, the orientation of cholesterol in the pocket is opposite to the conformation normally assumed by cholesterol in the membrane [222], with the alkyl chain facing the polar head group of the phospholipids composing the membrane. The most plausible hypothesis is then that cholesterol diffuses laterally into membranes. Indeed, in the case of tetraspanins localized in the plasma membrane the presence of the extracellular loop would preclude the entrance of cholesterol from the extracellular space. Nevertheless, we cannot exclude that intracellular cholesterol arriving to the plasma membrane might be directly inserted into the pocket. However, CD63 is unique, compared to other tetraspanins, for its endosomal localization. Consequently, CD63 into ILVs has the same orientation than at the plasma membrane while CD63 in the delimiting membrane has an opposite orientation, with the extracellular loops in the lumen of MVEs.

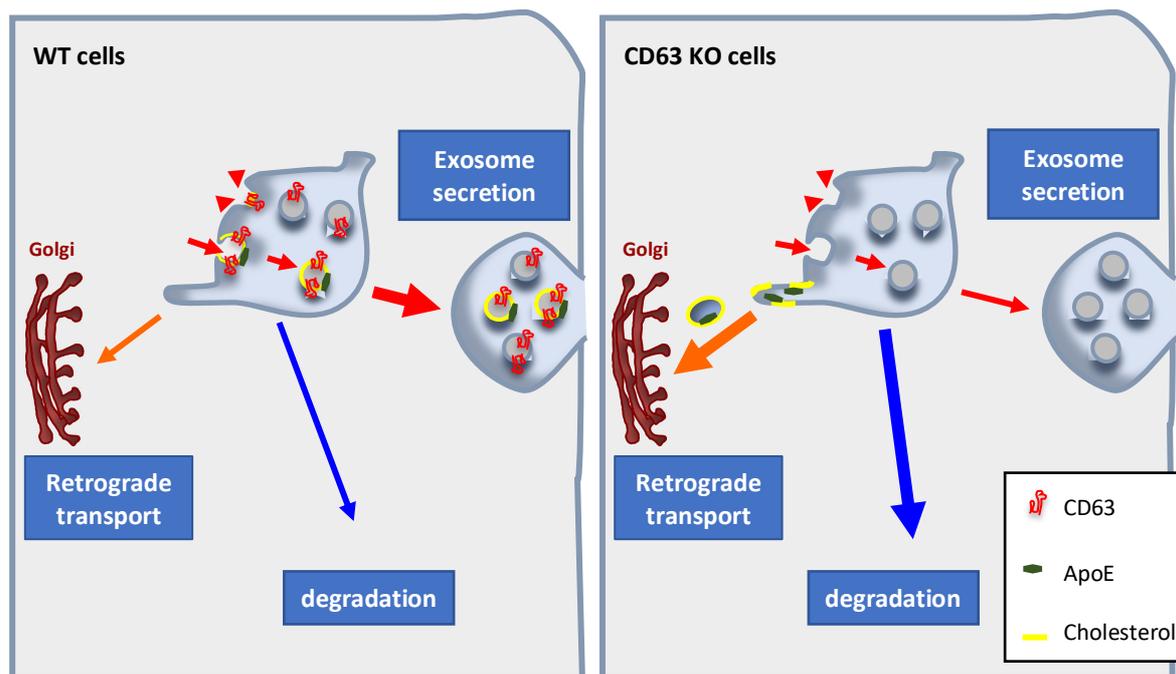
In a first scenario, newly synthesized cholesterol would be transferred to the delimiting membrane of MVEs and directly inserted into CD63 enriched microdomains. Here, newly synthesized CD63 is trafficked to the plasma membrane before being endocytosed to reach the endosomal system [314], therefore cholesterol might be transported to endosomes through endocytosis of pre-formed CD63-cholesterol enriched microdomains. The fact that CD63 has been found in caveolae, which are also cholesterol containing platforms [487] may support this idea. In a second scenario, I would propose that cholesterol associated to CD63 originates from the main external source of cholesterol for cells, LDLs, which are endocytosed and processed in late endosomes/lysosomes. LDL-derived cholesterol is normally transported to the delimiting membrane of endosomes by the cooperative action of NPC2 and NPC1. We cannot exclude that NPCs would contribute to enrichment of cholesterol in CD63 enriched domains on the delimiting membrane or to cholesterol loading of CD63 cavity.

It remains that CD63 may then mediate the sorting of cholesterol into ILVs, as shown by our study, or contribute to the egress of cholesterol from endosomes. Indeed, LDL-derived cholesterol has been shown to arrive in CD63 positive late endosomes and to egress from these endosomes to reach the plasma membrane through Rab8 positive but CD63 negative tubular carriers [378]. Despite the role of CD63 was not investigated in this study, CD63 could contribute to the lateral distribution of cholesterol on the delimiting membrane to allow cholesterol insertion into the tubular carriers. However, it should be considered that, contrarily to other late endosomal proteins such as Lamp1, CD63 is mainly localized in ILVs [325]. Similarly, cholesterol is highly enriched in ILVs [182]. Hence, CD63 could contribute to buffer MVE cholesterol content by efficiently sorting cholesterol into ILVs rather than controlling its endosomal retrieval.

Moreover, we cannot exclude that cholesterol would also contribute to the sorting of CD63 into ILVs. As suggested for tetraspanin enriched microdomains at the plasma membrane, cholesterol may stabilize CD63 enriched microdomains and stabilize the interaction of CD63 with other proteins or the maintenance of CD63 dependent cargoes into the microdomains. This might be the case for ApoE, which mostly associates with cholesterol containing lipoproteins and mediates cholesterol transport. In addition, MHC-II, whose secretion on exosomes is modulated by CD63 [328] and that localizes at the plasma membrane in CD63 enriched TEM [324], has been shown to bind cholesterol [488]. Other CD63 dependent cargoes (e.g. LMP1, Syt VII) are palmitoylated, hence their palmitate moieties, by binding cholesterol, may stabilize their localization in CD63 microdomains. Indeed, mutations in the palmitoylation sites of Syt VII impairs the formation of CD63-Syt VII complexes [331]. It would be interesting to modulate cholesterol endosomal content and analyze the localization of CD63 and its secretion on exosomes. Indeed, endosomal cholesterol content seems to influence exosome secretion. First, use of perfringolysin O to label cholesterol suggested that mostly MVEs that are enriched in cholesterol fuse with the plasma membrane to release exosomes [182]. Moreover, accumulation of endosomal cholesterol in oligodendroglial cells, induced by U18666A or NPC1 mutation or cholesterol loading,

enhanced the secretion of exosomes enriched of cholesterol [253]. Despite this study did not discriminate between enrichment of cholesterol in a single exosome and an increase in the total number of secreted exosomes, it is noteworthy that secretion of exogenously expressed CD63 was also increased. It should also be considered that different lipids, including sphingolipids, are accumulated in endosomes in case of NPC disease. Given the role of the sphingolipid ceramide in EV biogenesis, including the exosomal secretion of CD63 in HeLa cells [224] or in Oli-Neu cells [183], we cannot exclude that other lipids would also influence CD63 secretion. Nevertheless, the ceramide pathway is not required for the sorting of CD63 dependent cargoes in MNT-1 cells [139], [140]. Similarly, few studies reported that depletion of cholesterol using cyclodextrins increased exosomal release of specific cargoes such as viral proteins [489] or of the lectin-like oxidized LDL receptor [490]. However, it should be considered that cyclodextrins mostly deplete cholesterol from the plasma membrane but a more general effect on endosomal dynamics that in turn could affect exosome release has not been addressed yet. Finally, we observed that CD63 is present in both cholesterol positive and cholesterol negative MVEs, suggesting that different subpopulations of MVEs exist and that cholesterol is dispensable for CD63 sorting into ILVs. Similar observations were made in cells infected with EBV where CD63 was found in both low (with LMP1) and high cholesterol (with HLA-DR) containing MVEs [214]. However, LMP1 itself seem to redirect CD63 in cholesterol low MVEs that remain peripheric even after U18666A treatment. Hence in this case it is difficult to discriminate the consequences of LMP1 expression from the possible consequences of cholesterol unbalance.

In conclusion it is likely that CD63 and cholesterol together would organize specific subdomains on the delimiting membrane of endosomes which in turn will contribute to ILV formation, for instance by promoting membrane bending ([Figure 23](#)).



**Figure 23. Model of CD63 mediated endosomal processes.**

CD63 and cholesterol organize microdomains on the delimiting membrane of endosomes that induce membrane bending, promoting sorting of CD63 dependent cargoes (e.g. ApoE) into ILVs. This process in turn promotes ILV formation and exosome secretion.

In absence of CD63, retrieval of cholesterol and CD63 dependent cargoes is promoted. This process induces accumulation of cholesterol and ApoE in the Golgi apparatus, while the melanosomal protein PMEL is sorted for lysosomal degradation. Nevertheless, exosome secretion is not impaired, but such exosomes lacks CD63 dependent cargoes.

Overall, CD63 regulates exosome composition, not only by regulating cargo endosomal trafficking but also by determining the fate of MVEs by maintaining a balance between secretory pathways (exosomes, LROs) and degradative/recycling pathways (degradation in lysosomes or retrograde transport).

## 2. Roles of cholesterol

### 2.1. Endosome homeostasis, ILVs and exosomes

Our data show that cholesterol sorting into ILVs depends on CD63. But, why is cholesterol sorted into ILVs? As described in the introduction, cholesterol content of MVEs influences several processes of MVE maturation and fate. Hence, its level in the delimiting membrane of MVEs needs to be tightly regulated. The concentration of cholesterol on the delimiting membrane of MVEs can be sensed by ORP1L that in turn will determine MVE movement [272], [273]. While high cholesterol levels determine the transport of MVEs in the perinuclear region, promoting their fusion with lysosomes, low levels of cholesterol prevent their perinuclear transport. Hence, it can be hypothesized that MVEs with low cholesterol levels on the delimiting membrane might be fated for fusion with the plasma membrane.

Sorting of cholesterol into ILVs could then contribute to decrease cholesterol content on the plasma membrane. Consistent with this idea, cholesterol appeared to be enriched in ILVs of MVEs of which the delimiting membrane was mainly unlabeled. Such MVEs often fused with the plasma membrane releasing their ILVs as exosomes [182], [491]. Hence, cholesterol sorting into ILVs may be required to determine the fate of an MVE. Moreover, sorting of cholesterol into ILVs may represent a source of cholesterol for the cell. Therefore, when needed, cholesterol might be transferred from ILVs to the delimiting membrane of endosomes and from there to other cellular compartments. In this case NPC2 could extract cholesterol from ILVs and transfer it to the delimiting membrane of endosomes [371], [492]. Alternatively, back-fusion of ILVs with the delimiting membrane of endosomes could contribute to the redistribution of cholesterol on the delimiting membrane.

Once ILVs are secreted as exosomes, the presence of cholesterol in exosomes could contribute to their fate and functions. Indeed, in cellular membranes, enrichment of cholesterol in specific membrane subdomains, such as lipid raft, TEM and caveolae, contribute to membrane organization and fluidity. Therefore, we can hypothesize that different levels of cholesterol in exosomes would change their membrane rigidity. Use of fluorescent probes sensing membrane fluidity have suggested that exosome membrane is rigid and contain domains with ordered liquid phase [298], [493], [494]. Exosome membrane rigidity was also affected by pH changes [298], [493] and seem to correlate with the higher content of cholesterol (and other lipids) in these exosomes [298]. Consequently, by contributing to exosome membrane rigidity, cholesterol may have an impact on the fate of exosomes. For instance, studies conducted using synthetic nanoparticles with different elasticity have shown how this biophysical property can influence their in vivo biodistribution, their targeting to specific tissues and their uptake or their fusion with recipient cells (reviewed in [495]). Moreover, sorting of cholesterol into exosomes may promote the sorting of specific proteins that in turn would also influence the fate of such exosomes. Lastly, enrichment of cholesterol was observed in the case of exosomes that remained clustered on the cell surface upon secretion [247]. The enrichment of cholesterol may contribute to the recruitment of tetherin on such exosomes, that in turn modulates their clustering on the cell surface. However, it should be noticed that secretion of cholesterol-rich exosomes may be due to the use of Bafilomycin, that seem to redistribute cholesterol from the plasma membrane to endosomes [496] and that may promote secretion of MVEs fated for degradation.

In conclusion, cholesterol sorting into ILVs and future exosomes may contribute not only to the maintenance of endosome homeostasis but also to the function and fate of exosomes, once released in the extracellular environment.

## **2.2. Pigmentation and melanoma**

Maintenance of cholesterol homeostasis in melanocytes, to which CD63 contributes (**Results, section 2**), would be eventually important for melanocyte homeostasis and pigmentation. Nevertheless, very little is known. One study reported that cholesterol can

modulate melanogenesis by increasing the expression of known regulator of pigmentation (e.g. MITF) and by increasing melanin synthesis [497]. Moreover, treatment of melanocytes with U1866A induced the accumulation of the melanogenic enzyme tyrosinase in cholesterol-laden late endosomes/lysosomes which in turn decreased melanin synthesis [498]. Nevertheless, nothing is known about early stages of pigmentation. Given that the CD63 dependent pathway contributes to cholesterol sorting into ILVs of early melanosomes (**Results, section 2**) and that the same pathway is required for PMEL loading on ILVs, it is tempting to speculate that cholesterol on ILVs might contribute to PMEL fibril formation. Analysis of PMEL fibrils upon modulation of cholesterol endosomal content would help to answer this question.

As previously described, the levels of cholesterol in the delimiting membrane of endosomes can regulate the positioning and motility of endosomes through ORP1L protein [272], [273]. Interestingly, a similar function might be covered for melanosomes by melanoregulin, a protein involved in melanosome positioning and transport, which contain a cholesterol binding domain [499]. Therefore, sorting of cholesterol into ILVs of melanosomes may contribute to regulate the levels of cholesterol on the delimiting membrane of melanosomes that in turn would affect their motility and transfer to keratinocytes.

In the context of melanoma, dysregulation of cholesterol trafficking may in turn influence cancer progression. I have already mentioned that Rab8 vesicles transport cholesterol from CD63 positive endosomes to the leading edge of migrating cells, stimulating cell migration [378, p. 8]. Moreover, by regulating the localization of SNARE proteins, cholesterol influence the delivery of ECM components and integrins to the PM, that can also in turn regulate cancer cell migration. Of note, SNARE localization is regulated by cholesterol levels in the Golgi [402]. Hence, by changing cholesterol levels in the Golgi (**Results, section 2**), CD63 might indirectly impair cell migration. Finally, considering that CD63 and ApoE expression is reduced in metastatic melanoma [345], [456], cholesterol content of EVs released under these conditions may be changed and in turn impair EV functions.

### **3. ApoE trafficking, secretion and roles**

#### **3.1. Sorting of ApoE into ILVs is regulated by CD63**

Our study identified ApoE as a new cargo of the CD63 dependent ILV biogenesis pathway in melanoma cells and primary melanocytes (**Results, section 1 and section 2**). Of note, our attempts to prove a direct interaction of CD63 with ApoE were not successful (data not shown), supporting the idea that ApoE associates to CD63 enriched microdomains, likely for its affinity for the lipid composition of the domain and not because of a direct interaction with CD63 (**Figure 23**). Indeed, being a hydrophobic protein, ApoE is mostly associated with lipids as in the case of lipoproteins or in the case of cellular membranes. Despite the 3D structure of lipid-bound ApoE is not known, it is likely that ApoE would interact with the phospholipids of the lipid bilayer [403]. The enrichment of cholesterol in CD63 microdomains could then contribute to create a specific organization of the lipid bilayer for which ApoE has affinity. Nevertheless, it remains to be determined how cholesterol would influence the

association of ApoE to CD63 enriched domains. Analysis of ApoE secretion and cellular localization upon treatment with cyclodextrins, in order to deplete cholesterol, or loading of cholesterol into endosomes would contribute to elucidate this question.

### **3.2. New insights in the trafficking and secretion of ApoE**

Although an initial aim of my thesis was to further elucidate the intracellular trafficking of ApoE in melanocytes, we encountered some technical issues that did not allow us to completely answer this question. For instance, overexpression of ApoE-GFP in MNT-1 cells induced the formation of ApoE aggregates that accumulates mainly in ER structures and lipid droplet like structures. Despite we observed partial endosomal localization, we could not observe sorting of ApoE into ILVs, given that ApoE was only present on the delimiting membrane of endosomes. Hence, we did not perform live imaging of the ApoE GFP in order to study its dynamics. Moreover, we hypothesized that the sorting of ApoE into ILVs may be regulated by ApoE receptors. Given that mass spectrometry analysis of MNT-1 exosomes (not shown) identified the receptors sortilin and LRP-1 we focused our interest on them. Nevertheless, our results were not conclusive and mostly suggested that both sortilin and LRP-1 were not involved in the CD63-dependent trafficking of ApoE. Therefore, it is likely that ApoE is released from the Golgi apparatus through vesicular carriers and transported either directly to endosomes or to the plasma membrane and then reinternalized through endocytosis. Sorting into ILVs would then depend on the affinity of ApoE for the lipid composition of CD63 enriched domains. In absence of CD63, we propose that ApoE would follow cholesterol during its retrieval from endosomes to the Golgi apparatus.

Yet, we identified an alternative pathway of secretion for ApoE through exosomes in melanoma cells and primary melanocytes (**Results, section 1 and section 2**), identifying the CD63-dependent ESCRT-independent pathway as the biogenesis mechanism of ApoE-exosomes (**Results, section 1 and section 2**). Given that ApoE has been found on exosomes derived from different cell types [416]–[419], such pathway seems to be widespread. Moreover, staining of ApoE by immunoelectron microscopy (**Results, section 1**) shows that ApoE is on the outer membrane of exosomes with average size of 100nm-200nm. Nevertheless, we cannot exclude that a pool of ApoE is secreted through conventional secretory pathway or in association with lipoproteins. Floatation of MNT-1 exosomes on a bottom-up density gradient (**Results, section 2**) showed the enrichment of ApoE in a single exosome fraction. EM analysis of this fraction showed the presence of smaller exosomes (**Results, section 2**). Despite a lipid membrane was observed, such structures were in part reminiscent of lipoproteins. Use of cryo-electron microscopy, which preserve the native structure of lipid particles, would allow to further elucidate the 3D structure of these small exosomes. As shown in “**Results, section 1**”, use of cryo-EM allowed us to identify particular structures on the surface of exosomes that we called “caps”. Such caps consisted of multiple horizontal layers which created a closed cylindrical shape (**Results, section 1**) and were very reminiscent of lipoproteins [500], in which these layers seem to be due to the presence of

cholesterol and cholesteryl esters. However, we could not discriminate if ApoE would only be present in these caps or be associated with the exosome lipid bilayer. The fact that we did not observe such caps on exosomes derived from HeLa cells, which do not contain ApoE, suggests that caps are specifically associated to ApoE. In vitro studies have shown that when added to phospholipid vesicles, ApoE reorganizes the lipids to forms small discoidal or spheroidal ApoE-phospholipid complexes, that are reminiscent of HDL [403]. Hence, we can hypothesize that association of ApoE with a nascent ILV may induce remodeling of lipids to create the caps. Such process could be also favored by the presence of cholesterol in CD63 microdomains, that might contribute to a reorganization of the lipid bilayer. However, it cannot be excluded that other endosomal lipids, for instance derived from LDL degradation, would be captured by ApoE and contribute to the formation of the caps. Finally, in hepatocytes, recycling of ApoE derived from triglyceride-rich lipoproteins occurs in early endosomes and consist in the re-secretion of ApoE in association with HDL, a process that also promotes cholesterol efflux [501]. Hence, association of ApoE to exosomes in melanoma cells may represent an alternative pathway for cholesterol efflux.

### **3.3. Role of ApoE, MVEs and exosomes in physiological and pathological amyloid formation**

Our work shows that endogenous ApoE is required for the formation of PMEL amyloid fibrils in pigmented cells, hence contributing to the first step of pigmentation. PMEL amyloids represent an example of “functional” amyloids which serve physiological roles [128] and that, in contrast to pathological amyloids, are not toxic. Hence, the study of physiological amyloids could serve as a relevant physiological model to better understand pathological amyloids [502]. Indeed, the formation of PMEL fibrils in pigment cells and A $\beta$  fibrils from APP in Alzheimer’s disease share several features. PMEL and APP are both transmembrane proteins with a similar structure and following similar intracellular trafficking pathways. While APP is cleaved by the  $\beta$ -secretase BACE1 [503], PMEL is cleaved by its homolog BACE2 [134] and both are cleaved by a  $\gamma$ -secretase complex containing PSEN2 [136], [504]. Another common feature is the involvement of ApoE in both PMEL and A $\beta$  fibril formation. Despite the role of ApoE in the formation of A $\beta$  fibrils is not yet fully understood, it has been suggested that A $\beta$  oligomers are more toxic than A $\beta$  fibrils [505] and that ApoE association with A $\beta$  would stabilize A $\beta$  seeds and fibrils and slow down the formation of A $\beta$  fibrils from A $\beta$  oligomers [439], [440]. In the case of PMEL, our work showed that ApoE localized in early melanosomes, the cellular compartment in which PMEL cleavage occurs (**Results, section 1**). Hence ApoE, by promoting the loading of the luminal domain of PMEL into ILVs may favor the formation of stable fibrils that are not toxic. We also observed that both ApoE3 and ApoE4 were able to rescue the phenotypic defect of melanosomes observed in ApoE Knock-out mice (**Results, section 1**). However, ApoE4 was less efficient than ApoE3. In the case of A $\beta$  it has been shown that ApoE4 is less efficient in stabilizing A $\beta$  fibrils [441]. It can be then hypothesized that, in presence of ApoE4, PMEL fibril formation might be slower or that such

fibrils might be less stable. Hence, further investigation of the influence of different ApoE isoforms in PMEL fibril formation would be of interest.

The endosomal system also plays important roles in amyloid generation and clearance. First, early endosomes/maturing MVEs represent the site in which  $\beta$ -secretase would encounter and cleave APP [506]–[508], as also shown for PMEL [134]. Moreover, both PMEL and APP are sorted into ILVs. Interestingly, while PMEL sorting is CD63-dependent and ESCRT-independent [139], sorting of APP depends on the ESCRT machinery [506]. Such difference might also contribute to the non-toxicity of PMEL fibrils. In the case of PMEL, ApoE containing ILVs seem to act as amyloid-nucleating platforms, that may contribute to minimize the formation and exposure of possible toxic PMEL intermediates. This might not be the case for A $\beta$  fragments, given that sorting of APP into ILVs did not promote A $\beta$  generation [506]. Furthermore, it should be considered that while ApoE and PMEL are both produced by melanocytes, in the brain A $\beta$  is produced by neurons and ApoE is mostly produced by astrocytes, suggesting that A $\beta$  and ApoE would mostly encounter extracellularly.

Finally, exosomes and ApoE particles are implicated in the clearance of extracellular A $\beta$  peptides. Binding of ApoE to A $\beta$  mediates the uptake of these complexes through binding to ApoE receptors [430], followed by ApoE recycling and lysosomal degradation of A $\beta$  [443]. In a similar manner, exosomes binding to extracellular A $\beta$  aggregates may facilitate A $\beta$  uptake, contributing to the propagation of amyloids to other cells [509] or to the degradation of such aggregates [510]. Secretion of ApoE containing exosomes, that has been reported in the brain [416], [418], may also contribute to A $\beta$  clearance and to A $\beta$  fibrillation in the extracellular medium. Our study reported the release of ApoE containing exosomes by pigmented cells that also contained PMEL fibrils (**Results, section 1**), but, while secreted ApoE could exert specific function in the skin, the possible roles of secreted PMEL fragments are not known. Yet, our study strengthens the relevance of our physiological model of amyloid for the study of AD related processes.

In conclusion, a better understanding of the molecular machineries involved in the formation of physiological amyloids, to which this work also gave contribution, would definitely shed new lights on the roles of endosomes, exosomes and ApoE in the formation of pathological amyloids and contribute to the identification of new therapeutic targets in Alzheimer's disease.

#### **3.4. ApoE cellular and extracellular roles: melanocyte homeostasis and melanoma progression**

My work revealed new features and functions of ApoE in melanocytes and melanoma cells. Confirming previous studies [456], we found that at least some melanoma cell lines and primary melanocytes express and synthesize ApoE (**Results, section 1, 2 and 4**). In addition, we identify ApoE as a main regulator of melanocyte pigmentation, hence attributing a new tissue-specific physiological role to ApoE (**Results, section 1**). Moreover, we showed that,

similarly to other cell types [416]–[419], ApoE is released through exosomes (**Results, section 1 and section 2**).

Previous studies had identified ApoE, whose expression was lower in metastatic cell lines and in patients with metastatic cancer compared to non-metastatic cancer [456], [457], as a negative regulator of melanoma progression. ApoE secreted by melanoma cells seem to exert several roles that contribute to the inhibition of melanoma progression by acting on both melanoma cells and tumor microenvironment [456]–[458]. Nevertheless, the pathway of secretion of ApoE was not investigated. Our study suggests that exosomes may represent the main pathway of ApoE secretion by melanoma cells. Moreover, we investigated the possibility that, in addition to having an autocrine action, exosomal ApoE may interact with the extracellular matrix. Nevertheless, our data suggest that, contrarily to other EV subpopulations, ApoE positive exosomes do not interact with collagen, a main component of the ECM (**Results, section 4**). Although we cannot exclude that ApoE exosomes would interact with other ECM components, our observations also suggest that at least a pool of ApoE may remain in proximity of the secreting cells in order to exert an autocrine action on melanoma cells. Finally, additional hints about the role of ApoE may be obtained by modulation of ApoE secretion through CD63 depletion.

Interestingly, in melanoma, migrating cells are less pigmented compared to the primary tumor [511], suggesting a balance between pigment synthesis and migration. By exerting both cellular functions, as in the case of melanosome biogenesis (**Results, section 1**) [140], and extracellular functions, as in the case of melanoma progression [456], ApoE may contribute to maintain such balance. Hence, it is tempting to speculate that a tight regulation of ApoE expression, cellular localization and secretion is required to maintain melanocyte and skin homeostasis.

#### **4. EVs in melanoma progression**

##### **4.1. Melanoma EVs interact with collagen**

My study showed for the first time that EVs derived from different melanoma cell lines are able to physically interact with collagen, the most abundant fibrous component of the extracellular matrix. Unfortunately, so far, we have not been able to identify a specific EV component that would mediate this interaction. However, we or other groups have identified possible candidates in EVs. One protein that could mediate the interaction of EVs with collagen is fibronectin, a component of the extracellular matrix that we have found on EVs derived from the three melanoma cell lines analyzed in this study (**Results, section 4**). Fibronectin interact with collagen and seem to influence the organization of collagen fibers [512], [513]. Although our results were not conclusive, it is tempting to speculate that fibronectin on the surface of EVs may not only mediate the interaction with collagen but also remodel its organization. Our preliminary data suggest that collagen fibrillogenesis is impaired in presence of EVs (**Results, section 5**). Despite, collagen structure was not further characterized, it is tempting to speculate that fibronectin on the surface of EVs may contribute to this process. Moreover, the interaction of fibronectin containing EVs with

collagen have been suggested to modulate cancer cell migration by favoring the adhesion of migrating cells on collagen [473]. Our preliminary data suggest that melanoma EVs may have a similar role (**Results, section 5**). Nevertheless, our assay requires further optimization and a more precise mechanism needs to be determined. Other proteins that could mediate the interaction with collagen are integrins, which are collagen receptors largely found in EVs, including melanoma derived EVs [514]. Moreover, EVs bearing specific sets of integrins have been shown to determine organotropic metastasis [295]. Therefore, it can be speculated that once in specific organs, integrins may mediate EV interaction with the ECM, in addition to the interaction with specific cell types.

Our study also provided evidences that CD9 positive EVs bind collagen more efficiently than CD63 positive EVs (**Results, section 4**). Indeed, CD9 and CD63 positive EVs may represent two different subpopulations [174], hence they may have a different composition that in turn would influence their functions. Separation of CD63 and CD9 positive EVs by immunoprecipitation or their analysis by flow cytometry and mass spectrometry will help to better understand the composition of these vesicles in order to further study their specific functions.

Finally, considering the variability of ECM composition in different tissues, the study of the capacity of EVs to interact with the extracellular environment should be extended to other ECM components. This would allow to identify specific receptors and to further elucidate how EVs exert their functions in specific organs.

#### **4.2. Implication of EV biogenesis pathways in melanoma EV composition and roles**

As described in the introduction, EVs are a heterogenous group of membrane vesicles that differ for their size, density, biochemical and physical properties and composition. Such diversity is determined by their origin (endosome or plasma membrane) and by the mechanisms that regulate their biogenesis. Moreover, the recruitment of a specific sorting mechanism and their relative contribution to exosome biogenesis also depends on the cell type and cellular homeostasis and can be modulated during pathological conditions. In this regard, our study well illustrates how the exosomal secretion of a specific cargo (e.g. ApoE) can be regulated by a given molecular machinery and not by others (**Results, section 1 and section 2**). Furthermore, our analysis of different melanoma cell lines establishes the existence of subpopulations of EVs with different composition that may in turn impact on their functions (**Results, section 4**). Indeed, melanoma derived EVs have been shown to exert different functions during cancer progression, including angiogenesis, immune regulation and modification of tissue microenvironment. Moreover, EVs, likely with a different composition, can play opposite functions during cancer progression. Specific exosomal components are likely required for each function and their release into EVs can be certainly modulated during different phases of cancer progression. Hence, a better comprehension of EV biogenesis pathways and their regulation could be used to manipulate EV generation in pathological states such as cancer.

## CONCLUSIONS AND PERSPECTIVES

The results that I have obtained during my PhD illustrate a new role for the tetraspanin CD63 in regulating the sorting of cholesterol into exosomes. To our knowledge, this is the first report of a molecular mechanism supporting the sorting of a lipid into exosomes. CD63 would generate microdomains enriched in cholesterol and other CD63 dependent cargoes at the delimiting membrane of endosomes that could contribute to membrane bending, the first step of ILV generation. Nevertheless, CD63 alone is not sufficient to generate and release ILVs. Hence, the complete molecular mechanism needs to be determined. To this regard, the role of syntenin in this pathway is being investigated. Moreover, rescue of CD63 expression in CD63 KO cells will point out a specific role of CD63 in this pathway that is not compensated by other tetraspanins. Such pathway have a physiological relevance in pigmented cells, where CD63 regulates the sorting of ApoE and of the melanosomal protein PMEL [139], [140], a process required for skin pigmentation. Nevertheless, it remains to be determined how cholesterol would affect ApoE trafficking and PMEL fibril formation. Current experiments are aiming to answer this question. In addition, the physiological relevance of the CD63 dependent pathway can be investigated in vivo using the CD63 Knock-out mouse model.

Together with previous studies from the host laboratory and other groups, this work depicts CD63 as a general regulator of endosomal trafficking pathways and endosomal homeostasis. Our data show that CD63 would regulate not only ILV formation, but also intracellular trafficking pathways between the endosomal system and the Golgi apparatus and suggest that the retromer complex is implicated in such regulation. Current experiments are aiming to better elucidate the involvement of the retromer complex and its interrelation with CD63. Our current model proposes that CD63 and cholesterol organize specific microdomains on the delimiting membrane of endosomes in which protein cargoes fated for ILV sorting are accumulated and maintained, avoiding their lateral diffusion. While in pigment cells ApoE and PMEL are expected cargoes of this pathway, in HeLa cells no CD63-dependent cargo is known yet. For this purpose, comparative proteomic analysis of exosomes released from CD63 WT and KO cells may be of interest. Furthermore, we cannot exclude that, together with cholesterol, CD63 may regulate the sorting of other lipids, especially the ones enriched in tetraspanin enriched microdomains such as gangliosides. In this respect, lipidomic analysis of exosomes would be informative. Indeed, by being the constituents of EV membranes, lipids are main component of EVs. Nevertheless, so far, EV research has been mostly focused on the study of proteins and nucleic acids. Further studies are then needed to better understand the lipid composition of EVs, how it is influenced by pathological conditions and to determine the functions of these lipids. For instance, the lipid composition of EVs may influence their biophysical properties that in turn may determine the fate of EVs, such as their uptake or fusion with recipient cells. This hypothesis is currently being investigated by us in the frame of a collaborative work.

Lastly, our analysis of EVs from different melanoma cell lines identified the presence of subpopulations of EVs that may fulfill different functions. Such heterogeneity is indicative of the variety of biogenesis mechanisms by which such EVs originated.

In conclusion, given the importance of EV biogenesis mechanisms in determining EV composition and functions, a better knowledge of such pathways and their regulation, will definitely help to develop therapeutic approaches to modulate EV generation in pathological states such as cancer. On a similar line, a better understanding of EV composition during the different phases of cancer progression may be fundamental to use EVs as cancer biomarkers. Finally, a better understanding of the fate of EVs in recipient cells, process in which the endosomal system plays a central role, will be key for the development of EV-based therapies.

# **ANNEXES**



## Review Article

# To be or not to be... secreted as exosomes, a balance finely tuned by the mechanisms of biogenesis

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The release of extracellular vesicles such as exosomes provides an attractive intercellular communication pathway. Exosomes are 30- to 150-nm membrane vesicles that are generated in endosomal compartment and act as intercellular mediators in both physiological and pathological context. Despite the growing interest in exosome functions, the mechanisms responsible for their biogenesis and secretion are still not completely understood. Knowledge about these mechanisms is important because they control the composition, and hence the function and secretion, of exosomes. Exosomes are produced as intraluminal vesicles in extremely dynamic endosomal organelles, which undergo various maturation processes in order to form multivesicular endosomes. Notably, the function of multivesicular endosomes is balanced between exosome secretion and lysosomal degradation. In the present review, we present and discuss each intracellular trafficking pathway that has been reported or proposed as regulating exosome biogenesis, with a particular focus on the importance of endosomal dynamics in sorting out cargo proteins to exosomes and to the secretion of multivesicular endosomes. An overall picture reveals several key mechanisms, which mainly act at the crossroads of endosomal pathways as regulatory checkpoints of exosome biogenesis.

## Introduction

All cells, from bacterial to human, release different types of extracellular vesicles (EVs), which can be taken up by nearby or distant cells, modulating their activity. Hence, EVs can act as intercellular mediators in many physiological and pathological situations, including development, physiological communication (e.g. neurons and skin), immune response, cancer progression and metastasis, cardiovascular and neurodegenerative diseases [1]. The origin, nature, size and content of these vesicles are diverse. Despite the use in the literature of different terminology to identify EVs and debatable nomenclature, two main classes of EVs can be identified: microvesicles and exosomes [2]. Microvesicles are generated by outward budding and fission of the plasma membrane (PM) and the subsequent release of these vesicles into the extracellular space [3]. Their size usually ranges from 50 to 1000 nm in diameter, although they can become larger (up to 10  $\mu$ m) in the case of oncosomes. Exosomes originate as intraluminal vesicles (ILVs) via inward budding of the limiting membrane of maturing endosomes, which are usually referred to as multivesicular endosomes (MVEs) or multivesicular bodies (MVBs). MVEs can fuse with the PM and release ILVs as exosomes of diameter 30–150 nm into the extracellular environment [4]. Although an increasing number of studies have investigated the roles of EVs in cell–cell communication, less attention has been paid to the mechanisms involved in their biogenesis [5] and their regulation of EV secretion in the producing cells.

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In the present review, we focus only on exosome biogenesis, generation and secretion of MVEs (further details on microvesicle generation can be found in a more detailed review [3]). MVEs are part of the endosomal pathway allowing cargo proteins to be either recycled or degraded after internalization [6]. Therefore, an MVE cannot be considered only as the site of generation of future exosomes, but rather as a dynamic maturing compartment with various functions that result from the coordination of multiple intracellular molecular mechanisms. The endosomal pathway has several crossroads that allow either the recycling of cargo proteins to the PM or to the Golgi apparatus or their sorting to ILVs. MVEs form a crossroad targeting cargo proteins that are sorted on ILVs to either secretion or lysosomal degradation. Each sorting step and each crossroad in the endosomal pathway can thus be viewed as a regulatory checkpoint. As a consequence, the different mechanisms involved in exosome biogenesis, from intracellular trafficking of cargo proteins to MVEs, including sorting into ILVs and targeting MVEs to the plasma membrane, act as regulatory elements of exosome biogenesis. We discuss how each step of the endosomal pathway can influence exosome biogenesis, with a particular focus on the balance between endosomal degradation and secretion.

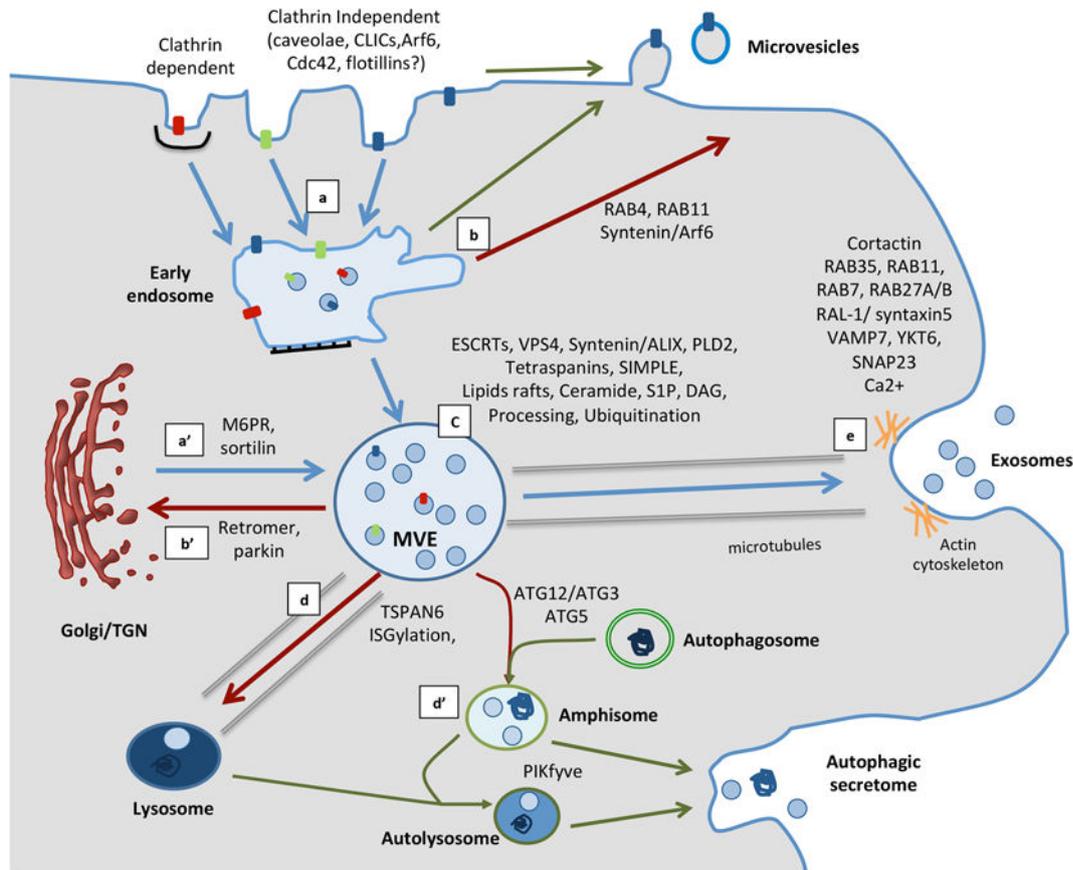
## Expression of cargo proteins as the first regulator of EV biogenesis

Expression of cargo proteins destined for exosomal secretion can be viewed as the first regulators of exosome formation because the targeting of a particular protein cargo to MVEs can induce the recruitment of specific machineries leading to ILV formation. This idea is supported by the observation that ectopic expression of MHC class II in HeLa cells [7], or its forced expression in intestinal epithelial cells after stimulation by interferon  $\gamma$  (IFN- $\gamma$ ) or expression of its transactivator CIITA, increased the release of EVs [8]. This increased secretion can be attributed to the increased generation of MVEs and the recruitment of sorting machineries by the cargo protein, which promote ILV (or future exosome) generation. Expression of another exosomal cargo protein in HeLa cells, PMEL, a specific melanosomal protein, induces sorting to ILVs via recruitment of specific sorting mechanisms [9], generation of subpopulations of ILVs [10] and production of exosomes (G. van Niel, unpublished data; [10,11]). Similarly, overexpression and depletion of syndecan from MCF-7 cells increase and decrease the recovery of exosomal markers respectively [12]. In this context, regulation of the expression of a given cargo protein, which is either cell type specific or induced by external stimuli, is the first regulatory element to modulate exosome biogenesis. It should be noted that a default in exosome secretion could then reflect impaired expression of exosomal cargo proteins. Alternatively, such a default could result from inhibition of MVE secretion or a defect in ILV generation. To avoid such misinterpretation, cargo protein expression, exosome recovery and exosome generation as ILVs in MVEs should be investigated in parallel. Isolation and methods of investigation used for the study of EVs can lead to selection or enrichment of a given subpopulation of exosomes [13]. Therefore, particular attention should be also paid to the methods and cellular models used in each study for a clear conclusion of whether there is a defect of ILV generation or a defect of MVE secretion.

## Targeting cargo proteins to MVEs

To be secreted on exosomes, cargo proteins need to reach the endosomal pathway before being sorted into ILVs. Mutated versions of proteolipid protein (PLP) retained in the endoplasmic reticulum, for example, cannot reach the endosomal pathway and are not secreted on exosomes [14]. Exosomal membrane cargo proteins can reach endosomes by becoming internalized from the PM (see Figure 1a) or by direct targeting from the Golgi body (see Figure 1a').

Known cargo proteins of exosomes, such as premelanosome protein (PMEL) [9] and amyloid precursor protein (APP) [15], are first transported to the plasma membrane and from here they are internalized to reach the endosomes. Endocytosis of proteins can involve different pathways, the most frequently studied being clathrin-dependent endocytosis, which requires polymerization of a clathrin coat on the cytoplasmic face of a curved plasma membrane and subsequent invagination and scission of the vesicles; with this last step mediated by the GTPase dynamin [16]. Notably, this pathway is used by mature MHC-II to reach Arf6<sup>+</sup>Rab35<sup>+</sup>EHD1<sup>+</sup> tubular endosomes [17] and by epidermal growth factor receptor (EGFR) [18]. It is of note that such endocytosis depends on ubiquitination of the cargo proteins [19,20], targeting them for degradation rather than exosomal secretion. Other pathways, defined as 'clathrin-independent endocytosis' (CI), include caveolae-mediated endocytosis (also dynamin dependent) and the clathrin- and dynamin-independent carrier (CLIC) tubular intermediates [21]. Such pathways allow internalization of different cargo proteins such as CD44 and glycosyl phosphatidylinositol (GPI)-anchored protein [22,23] or galectin-3 [24], which are all present on exosomes. The relevance of the CI pathway in exosomal targeting of these cargo proteins has not yet been proved, although small GTPases such as the ADP-ribosylation factor 6 (Arf6) and Rho subfamily members RhoA and Cdc42, which have been implicated in the regulation of different pathways of CI endocytosis, are all found in EVs; in addition, Arf6 and Cdc42 can modulate exosome and microvesicle secretion [25-27]. Finally,

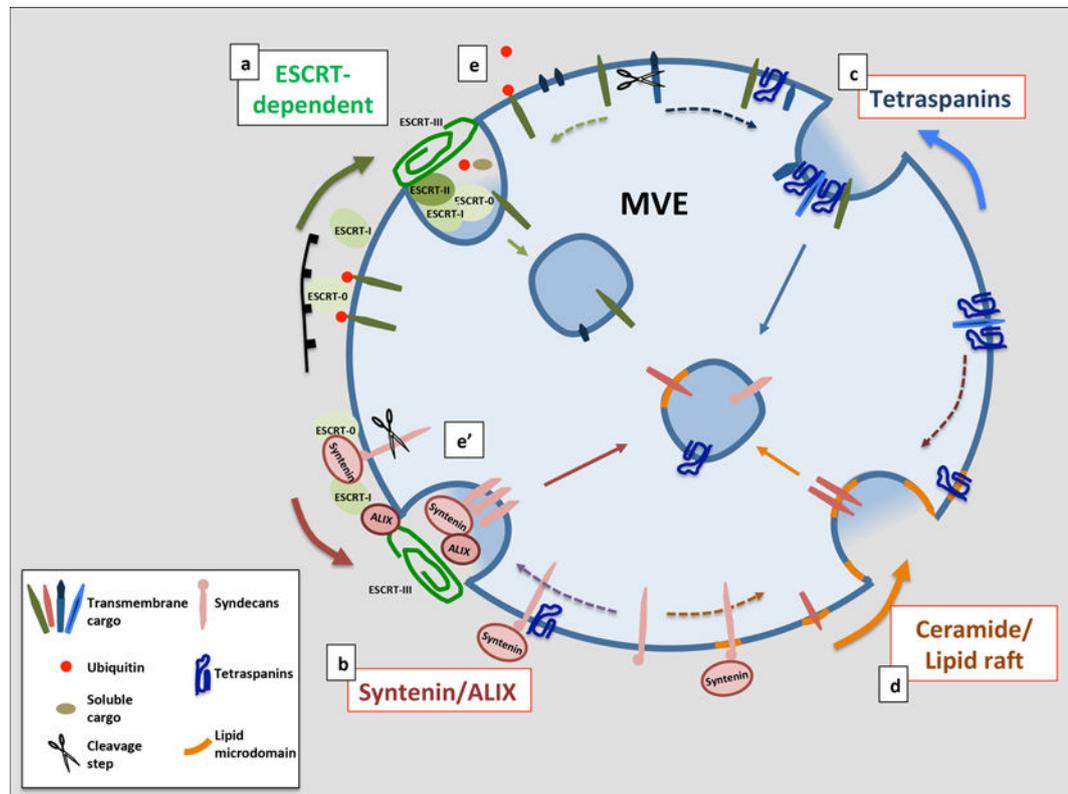


**Figure 1. Intracellular trafficking checkpoints involved in exosome biogenesis and secretion**

The letters in white squares indicate the different checkpoints. The blue arrows highlight the trafficking steps favouring exosome biogenesis and the red arrows highlight the trafficking steps impairing exosome biogenesis. The green arrows represent alternative release mechanisms (microvesicles and autophagic secretion). Exosome biogenesis and release are regulated by multiple checkpoints that are annotated by the letters 'a' to 'e'. Exosomal cargo proteins reach the endosomal compartment (a) through endocytosis or (a') by direct transport from the Golgi body. Before being sorted into ILVs, the cargo proteins need to avoid recycling to (b) the PM or (b') the Golgi body. (c) Once formed (see also Figure 2), MVEs have to escape different fates such as fusion with (d) lysosomes or (d') autophagosomes, and be transported towards the PM in order (e) to fuse with it and secrete exosomes into the extracellular environment. The main reported regulators of each step are indicated at each regulatory step.

flotillins, which are commonly used as exosome markers, can regulate endocytosis of specific cargo proteins, although the underlying mechanisms are still poorly understood. Flotillins may favour the clustering of cargo proteins, thereby promoting their endocytosis, which in some cases may require the presence of clathrin [28]. All these internalization pathways will deliver their cargo proteins to early endosomes (EEs) [6], providing a first checkpoint (see Figure 1a) for targeting cargo proteins on MVEs and exosomes.

Early endosomes appear as vacuolar compartments, which are characterized by the presence of the small GTPase RAB5, from which thin tubular structures are pulled to generate recycling endosomes (REs). Cargo proteins targeted to recycling endosomes are targeted back to the PM (see Figure 1b) whereas those destined for MVEs will accumulate on vacuolar subdomains of the EEs and be sorted into ILVs. Hence, cargo proteins that are destined for recycling to the PM will probably not be enriched in exosomes, unless their recycling is impaired, as in the case of the transferrin receptor in reticulocytes [29]. Such a balance between recycling and targeting to MVEs can be seen as a second checkpoint (see Figure 1b) regulating exosome biogenesis. Among the proteins that regulate this crossroad between recycling and intraluminal sorting, the PDZ protein syntenin is an ideal candidate. In endosomes, syntenin interacts with syndecans to support their recycling to the PM via a mechanism requiring production of phosphatidylinositol 4,5-bisphosphate PI(4,5)P<sub>2</sub> and the small GTPase Arf6 [30]. Alternatively, syntenin, together with the endosomal sorting complex required for transport (ESCRT) accessory protein ALIX, can dispatch syndecans to ILVs (see Figure



**Figure 2. Sorting mechanisms at MVEs involved in exosome biogenesis**

The bold arrows outside the MVEs indicate the main sorting mechanisms reported so far. The dashed arrows within the lumen of the MVEs indicate a potential intersection or coordination between sorting mechanisms. Four main sorting mechanisms have been reported so far for transmembrane proteins: (a) the classic ESCRT mechanism, (b) the syntenin–ALIX pathway, (c) the tetraspanin microdomains and (d) the ceramide pathway. (e,e') Post-translational modifications are key regulators, allowing controlled targeting of a modified cargo protein (ubiquitination, cleavage) to a specific sorting mechanism.

1c) [12]. Syndecans, syntenin and ALIX assemble in a tripartite complex which can drive not only their own exosomal release, but also the release of CD63 and HSP70. It is of interest that Arf6 and its effector PLD2 also control budding of ILVs and biogenesis of exosomes via the syndecan–syntenin–ALIX pathway [27]. Although it still has to be determined whether recycling of syndecans directly affects syntenin–exosome biogenesis, the fact that the same proteins play a role in both recycling and intraluminal sorting clearly illustrates the role of EEs as the first ‘sorting station’ of the cell, in which the fate of a cargo protein is primarily decided. It could then be proposed that the ‘internalization’ and ‘recycling’ checkpoints, by regulating localization of cargo proteins between respective sites of exosome and microvesicle generation, i.e. MVE or PM, may modulate a potential balance between exosome and microvesicle biogenesis. In this context, Arf6, by being involved in both exosome [27] and microvesicle [26] biogenesis, may control the balance between the generation of both subpopulations of EVs.

Direct transport of proteins from the Golgi apparatus to the endolysosomal system has been mostly studied for the transport of lysosomal proteins. Transmembrane proteins possess a small amino acid motif (i.e. tyrosine- or dileucine-based motifs), which will be recognized by clathrin adaptor proteins, allowing the formation of Golgi-derived, clathrin-coated carriers that then fuse with the endosomes [31] (see Figure 1a'). The relevance of direct transport from the Golgi apparatus to MVEs and ILVs is illustrated by the protein GPRC5, a member of the G-protein-coupled receptor (GPCR) family proteins, which uses GGA1 carrier vesicles for sorting on exosomes [32]. Soluble proteins must bind specific receptors (e.g. mannose phosphate receptor, sortilin) for transport from the Golgi body to MVEs. Once in the endosome, the cargo proteins are released whereas the receptors usually recycle back to the trans-Golgi network by retrograde transport [31]. Retrograde transport can then be seen as an alternative exit route from endosomes which cargo proteins should avoid to end up in ILVs (see Figure 1b'). This additional cross-road represents a third checkpoint (see Figure 1a' and Figure 1b') that could be regulated by parkin, a mutated E3

ubiquitin ligase found in Parkinson's disease. Parkin would regulate such a balance between retrograde transport and targeting to ILVs by acting on both retrograde transport (through the retromer complex) and inward budding, and exosome secretion [33]. In addition, sortilin, a receptor involved in anterograde transport from the Golgi body and a known cargo protein of retrograde transport [34], has also been shown to be secreted on exosomes and to modulate exosome biogenesis [35], although the trafficking mechanisms underlying such regulation need further investigation.

## ILV biogenesis in MVEs

Exosomes are generated as ILVs in maturing endosomal compartments, providing a central checkpoint in this pathway (see Figure 1c). The morphological changes induced by the generation of ILVs, which give MVEs their multivesicular appearance, are associated with the maturation of early endosomes into late endosomes; this requires switching of the Rab GTPase RAB5 with RAB7 [6], which can be blocked by overexpression of a constitutively active mutant form of RAB5(Q75L). This mutant induces fusion of early endosomes that can still form ILVs despite their enlarged appearance [14]. For this reason, RAB5 mutant-expressing cells have often been used to visualize ILVs and to study the sorting of cargo proteins to ILVs [12,14,36,37]. In addition, depletion of RAB5 in HeLa cells [7] or RAB7 in MCF cells [12] (but not in HeLa cells [7]) has been shown to modulate exosome secretion, although they may have an overall effect of endosome homeostasis rather than a direct role in exosome biogenesis. It is of note that the precise step of MVE maturation at which they would be preferentially secreted is still unknown.

Within maturing endosomes, probably alongside the RAB5/RAB7 switch, future exosomes are generated as ILVs through a process requiring the invagination and fission of the endosomal membrane to form an ILV (see Figures 1c and 2). The first mechanism to be described for this process was the ESCRT dependent one (see Figure 2a). The ESCRT complex is composed of four different subcomplexes – ESCRT-0, -I, -II and -III – and associated proteins such as ALIX, VTA1 and ATPase VPS4 [38]. These subunits act together in a stepwise process, starting with recruitment of ESCRT-0 at the endosomal limiting membrane, through ubiquitin moieties attached to the cytoplasmic domain of the transmembrane cargo proteins that have to be sorted. Importantly, the cargo protein [and phosphatidylinositol 3-phosphate (PI<sub>3</sub>P)] recruits the sorting machinery, strengthening the notion that it is the first regulator of ILV biogenesis. ESCRT-0 and ESCRT-I then cluster the cargo proteins under a flat clathrin coat, creating a subdomain of the endosomal membrane that buds into an ILV. The flat clathrin coat is thought to avoid cargo protein diffusion, and possibly their targeting to alternative pathways such as recycling or retrograde transport [39]. This coat must be removed, potentially by the exosome-enriched protein V-ATPase HSC70 [40,41], to allow the recruitment of ESCRT-II and -III, the latter being responsible for membrane scission, together with VPS4. During this process, ESCRT-III recruits deubiquitinating enzymes that remove the ubiquitin tag from cargo proteins before the release of newly forming ILVs into the lumen of MVEs. However, ubiquitinated proteins can be still found in exosomes, suggesting that deubiquitination is not a critical step in exosome biogenesis [42].

The role of the ESCRT complex has been deeply investigated by Colombo et al. [43] using RNA interference screening that targets 23 ESCRT and ESCRT-associated proteins in HeLa cells; this study identifies 7 proteins that affect exosome secretion. Although depletion of ESCRT-0 or -I components STAM, HRS or TSG101 reduced exosome secretion, depletion of ESCRT-III CHMP4C, VPS4 and accessory molecules VTA1 or ALIX increased it. It should be noted that, in the same study, depletion of ALIX increased MHC-II secretion on exosomes but had variable effects on CD63 and HSC70 levels, suggesting that ALIX may have more effect on the composition of exosomes than on overall exosomal secretion. As the study mainly focused on the analysis of exosomes once they had been released from HeLa cells expressing the CIITA transactivator, it is still unclear which steps of exosome biogenesis were affected and whether they are specific to the forced expression of MHC-II. However, the ESCRT-0 protein HRS has also been involved in exosome secretion in dendritic cells [44] and the release of exosomal Wnt3 [36], and its depletion was shown to decrease overall exosome number, as measured by nanoparticle tracking analysis [45]. More recently, inhibition of VPS4 in HEK293 cells has been shown to decrease release of different subpopulation of EVs, corresponding to CD63-enriched exosomes and CD9-enriched microvesicles [46]. It is of interest that electron microscopy has shown that HeLa cells, depleted for HRS, display MVEs containing small ILVs (<40 nm in diameter) [10]. Therefore, it cannot be excluded that some subpopulations of exosomes, due to their small size, are below the detection threshold of some investigative methods (such as nanoparticle tracking analysis) or still lack specific markers for detection by Western Blot. Complementary approaches are therefore recommended for investigating exosome subpopulations that have been secreted.

The ESCRT mechanism can be partially (via ALIX; see Figure 2b) or completely dispensable for the generation of ILVs. In this context, to identify ESCRT components involved in syndecan–syntenin–ALIX exosome biogenesis, Baietti et al. [12] performed RNA interference screening on MCF-7 cells. Depletion of TSG101 (ESCRT-I), VPS22

(ESCRT-II), CHMP4 (ESCRT-III) or VPS4 reduced exosomal release of syndecans, syntenin and CD63, suggesting that these components are involved in this pathway. In contrast to the conventional ESCRT pathway, however, ALIX could provide an alternative route by recruiting CHMP4 and VPS4 directly. It should be noted that this study mostly analysed the specific syndecan–syntenin exosome subpopulation in MCF-7 which would explain the discrepancies with other reports investigating similar processes in HeLa cell CIITA [43]. Hence, the preferential mechanism for biogenesis can vary depending on the cell type and the cargo proteins enriched in specific subpopulations of exosomes.

ESCRT-independent biogenesis of ILVs was first revealed using simultaneous depletion of components of the four ESCRT subcomplexes [47]. Electron microscopic analyses have shown that ESCRT-independent ILVs are enriched in the tetraspanin CD63 [10,47]. Tetraspanins are a family of proteins with four transmembrane domains. They share a similar structure and organize as highly dynamic membrane microdomains known as tetraspanin-enriched microdomains (TEMs), in which tetraspanins interact with each other and with other transmembrane or cytosolic signalling proteins [48]. Different tetraspanins have been proposed to have a role in ILV formation (see Figure 2c). Bone marrow dendritic cells (BMDCs) from CD9 knockout mice secrete fewer exosomes compared with wild-type mice [49], whereas expression of CD9 and CD82 promotes the release of  $\beta$ -catenin in exosomes [49]. In a mouse model of breast cancer, the release of Wnt1 on exosomes from cancer-associated fibroblasts depends on the tetraspanin CD81 [50]. Expression of another tetraspanin, Tspan8, has been shown to change exosomal protein and mRNA content in rat adenocarcinoma cells [51]. Finally, the tetraspanin CD63, which is particularly rich in exosome membranes, plays an important role in exosome biogenesis in multiple cell types. CD63 is required for the generation of small ILVs in HeLa cells depleted for HRS [10] and has recently been involved in the biogenesis of exosomes in fibroblasts from patients with Down's syndrome [52]. CD63 associates with and targets the Epstein–Barr virus (EBV)-encoded latent membrane protein 1 (LMP1) to ILVs; it is critical for its secretion on exosomes [53,54]. In melanocytes, CD63 is required for the sorting of the melanosomal protein PMEL to ILVs [55], a process involving the targeting of apolipoprotein E (ApoE) to ILVs and exosomes, but not the ESCRT complex [56].

In addition to proteins, lipids have also been involved in exosome biogenesis (see Figure 2d). The first ESCRT-independent mechanism reported for exosome biogenesis was identified in Oli-neu cells; it requires generation of the sphingolipid ceramide via hydrolysis of sphingomyelin by neutral type II sphingomyelinase [14]. Ceramide could create specific lipid microdomains and induce negative membrane curvature, which give rise to ILVs and exosomes enriched in ceramide. In addition a metabolite of ceramide, sphingosine 1-phosphate, has recently been shown to continuously activate  $G_i$ -protein-coupled sphingosine 1-phosphate receptor and in this way to regulate the sorting of cargo proteins into exosomal ILVs [57]. Exosomal membranes can also contain lipid rafts, specific lipid microdomains enriched with cholesterol and glycosphingolipids, which can contribute to ILV formation and sorting of raft-associated molecules such as GPI-anchored proteins [58]. In a similar way, the  $Ca^{2+}$ -dependent, phospholipid-binding protein, annexin A2, binds to the lipid raft and is transported along the endocytic pathway, where it is sorted into ILVs [59]. Diacylglycerol (DAG), another lipid second messenger, also seems to have a role in exosome formation. In fact inhibition of DGK, an enzyme that metabolizes DAG into phosphatidic acid, has been shown to induce release of exosomes in T-cells [60]. Finally, bis(monoacylglyceryl)phosphate (BMP), formerly termed 'lysobisphosphatidic acid', is specifically enriched in late endosomes such as MVEs and can recruit ALIX to generate ILVs *in vitro* and *in vivo* [61]. Phospholipase D (PLD), which hydrolyses phosphatidylcholine to generate choline and phosphatidic acid, has also been involved in the generation of exosomes via the syntenin–ALIX pathway [27]. Lipids are therefore major actors in exosome biogenesis and their contribution requires further investigation to be fully understood.

The overall picture of the sorting step at MVEs (see Figure 2) involves several sorting mechanisms that are not necessarily mutually exclusive. The proteolipid interactions, which are well known to induce membrane curvature [62], are a first example of the interconnections between the multiple sorting mechanisms acting at MVEs. A potential example is a recent structural study of the tetraspanin, CD81, which revealed a cone-like structure with an intramembrane pocket that can bind cholesterol [63]. As proposed for the cone-shaped ceramide [14], clustering of several cone-shaped tetraspanins could then induce inward budding of these microdomains. Another interrelationship between sorting mechanisms is provided by the known interaction of CD63 with syntenin [64], the regulation of CD63 by the ALIX–syntenin pathway [12], and the formation of highly dynamic microdomains by different tetraspanins such as CD63, CD9 and CD81 [65,13]. Moreover, the syntenin–ALIX pathway in MCF cells [12] and the sorting of CD63 in HeLa cells [66] are both regulated by ceramide. Interconnections between the reported sorting mechanisms seem to be highly relevant for exosome biogenesis but also to depend on the cell type under investigation, as exemplified by the sorting of PMEL through ApoE, a soluble apolipoprotein with a particular affinity for lipids, which depends on CD63 but not on ESCRT or ceramide [56] in pigment cells.

There is also a similar intersection or coordination between ESCRT-dependent and -independent mechanisms. Non-ubiquitinated cargo proteins can be trapped in microdomains formed by the clustering of the highly ubiquitinated tetraspanin protein Cos. This ubiquitination ‘in *trans*’ allows the recruitment of the ESCRT mechanism and ‘canonical’ formation of ILVs in which non-ubiquitinated cargo proteins are also sorted [67]. ESCRT-dependent and -independent mechanisms for ILV biogenesis are tightly coordinated on the same MVE by the tetraspanin CD63 in pigment cells [55]. It is rather unlikely, however, that all sorting mechanisms act on the formation of a single ILV because ESCRT-dependent and -independent mechanisms give rise to a heterogeneous population of ILVs in terms of both size and cargo proteins [10]. The variety of sorting mechanisms in a single cell type would then contribute to the heterogeneity in size and composition of exosome subpopulations.

Overall, both ESCRT-dependent (at least ESCRT-0 and -I which are involved in the syntenin–ALIX pathway) and -independent mechanisms can participate to a different extent in exosome biogenesis, and provide a central checkpoint in this pathway (see Figure 1c). ESCRT-independent and semi-dependent mechanisms (such as the syntenin–ALIX pathway) seem nevertheless to favour ILV release as exosomes while the conventional ESCRT-dependent mechanisms seem more dedicated to degradation. The reasons for the connection between sorting mechanisms and fates of MVEs are still unknown, but several observations described below provide support.

## Modification of cargo proteins for sorting to exosomes

As cargo proteins are the main recruiters of sorting mechanisms at MVEs, any modification of cargo proteins destined for exosomal release may determine their sorting and release on exosomes. A major regulator of the sorting of proteins to exosomes is their post-translational modification (PTM) (see Figure 2e and e’). Different PTMs can compete to determine the fate of the same cargo protein [68]. A major PTM is ubiquitination (see Figure 2e) which consists in binding ubiquitin moieties to cysteine residues present in the cytoplasmic domain of a transmembrane protein, or present and accessible on soluble proteins. Its role in the sorting of cargo proteins into ILVs destined for lysosomal degradation has been extensively studied and involves the recognition of the ubiquitinated cargo proteins (e.g. EGFR, MHC-II) by the ESCRT mechanism. Furthermore, ubiquitinated MHC-II is targeted to MVEs destined for lysosomal degradation and sorted to ILVs probably via an ESCRT-dependent mechanism. On the contrary, non-ubiquitinated MHC-II can be secreted on exosomes and sorting to ILVs involves incorporation into CD9-containing membranes, probably in an ESCRT-independent manner [69]. Similarly, exosomal secretion of SIMPLE [70] is enhanced after mutation of its binding site for ubiquitin ligase, suggesting that ubiquitination negatively regulates SIMPLE secretion on exosomes. This set of studies strengthens the notion that ESCRT-dependent and -independent mechanisms are respectively associated with exosome release and lysosomal degradation.

Other PTMs that could be important for the incorporation of cargo proteins into exosomes are the addition of a small ubiquitin-related modifier (SUMO), as reported for the heterogeneous nuclear ribonucleoprotein A2B1 (hn-RNPA2B1), a key regulator for the recognition of specific miRNAs and their loading into exosomes [71], phosphorylation, as in the case of annexin A2 [59], and oxidation, as in the case of  $\gamma$ -synuclein [72]. Moreover the detection of many glycosylated proteins in exosomes suggests that glycosylation may also have a role in exosomal sorting, despite the need for further investigation [68]. Finally, ISGylation, the addition of the small ubiquitin-like protein ISG15, has recently been shown to negatively regulate exosome release [73], although it would act on the sorting mechanism and the fate of the MVE rather than on the cargo protein itself.

In addition to PTM, cargo proteins can undergo proteolytic cleavage before being incorporated into exosomes (see Figure 2e’). Heparanase, the only mammalian enzyme able to cleave heparan sulphate internally, was identified as a stimulator of the syndecan–syntenin–ALIX pathway [74]. Indeed, endosomal heparanase trims the heparan sulphate side chains of syndecans. Such trimming would favour clustering of syndecan and its proteolytic processing to generate ‘SDC-CTF (syndecan C-terminal fragment)’, which is sorted to ILVs. Such processing may reinforce the syndecan–syntenin interaction, thereby favouring ILV budding. Similar coordination between processing and sorting is well illustrated in melanocytes with the protein PMEL [75]. PMEL is cleaved by the secretase BACE2 at the limiting membrane of pigment cell-specific MVEs. The two cleavage products of PMEL, its luminal domain and PMEL-CTF, are taken care of by distinct sorting mechanisms within the same compartment. While PMEL-CTF is sequestered in an ESCRT-dependent manner into the clathrin coat at the limiting membrane of the compartment, and destined for lysosomal degradation, the luminal amyloidogenic domain of PMEL is loaded on the surface of ILVs [55] and can be secreted on exosomes [56] in a CD63-dependent manner. Although the role of CD63 in loading the luminal domain of PMEL into ILVs is still not completely clear, depletion of CD63 induces the targeting of full-length PMEL to the ESCRT-dependent pathway, suggesting that this tetraspanin is a key coordinator of the interrelated processing and sorting of cargo proteins destined for exosomal release.

It should be expected that the recruitment and contribution of these different sorting pathways to exosome biogenesis depend not only on the PTM or processing of the cargo protein to be sorted but also on the cell type, cellular homeostasis and pathological conditions. However, any enzymes involved in the modification or processing of the exosomal cargo proteins could be considered as a regulator of exosome biogenesis which would complete the checkpoint controlling ILV biogenesis (see Figure 1c).

## Balance between degradation and secretion of MVEs

The main fate of an MVE is to fuse with lysosomes and degrade their content. Therefore, MVEs must avoid lysosomal degradation in order to release their ILVs as exosomes. This step is also a critical checkpoint particularly relevant in several pathologies (see Figure 1d). Several studies in which inhibition or blocking of lysosomal function induces exosome secretion support the hypothesis of a balance between lysosomal degradation and exosome secretion. For instance, treatment with bafilomycin A1, a lysosome inhibitor, increases EV secretion [73,76] or exosomal secretion of specific cargo proteins such as  $\alpha$ -synuclein [77]. On the same line, inhibition of V-ATPase in *Caenorhabditis elegans* has been shown to trigger apical secretion of the Hedgehog protein [78]. Such a balance seems to be highly relevant for pathological conditions in which lysosome activity is affected, such as lysosomal storage diseases or neurodegenerative diseases, e.g. Niemann–Pick disease type C (NPC) is caused by a mutation in the cholesterol transporter NPC1 and characterized by the accumulation of cholesterol in endosomes and lysosomes. One study showed that NPC1 mutation or inhibition with drugs (U18666A) enhances secretion of cholesterol in exosomes [79]. Similar impairment of lysosome function is often a feature of neurodegenerative diseases and an intracellular accumulation of toxic proteins has been proposed to provoke their extracellular secretion [80]. Accumulations of APP fragments A $\beta$  and tau are typical features of Alzheimer's disease, and both proteins were found in EVs released from cell culture models of Alzheimer's disease, in EVs isolated from cerebrospinal fluid, brain extracellular space and blood samples from patients with Alzheimer's disease [81,82]. There is, so far, no direct evidence of a causal connection between intracellular accumulation and the release of these pathological proteins in EVs, but such a pathway would certainly compensate for their impaired degradation inside the cells, thereby contributing to the maintenance of cellular homeostasis. This set of studies also strengthens the first studies on exosomes [83] that showed exosomes in a way eliminating obsolete or potentially toxic compounds that had accumulated in cells.

Mechanisms that prevent lysosomal degradation in favour of exosome secretion provide a powerful checkpoint (see Figure 1d) for regulation of exosome biogenesis, but they still remain poorly understood [80]. As suggested previously, ESCRT-independent and -dependent sorting seems to drive MVEs to either secretion or lysosomal degradation respectively. New regulators of sorting mechanisms at MVEs reinforce their regulatory role in the balance between secretion and degradation. ISGylation of TSG101 [73] inhibits exosome secretion whereas mutations that impair ISGylation enhance exosome secretion. Such regulation is counterbalanced by the fusion of MVEs with lysosomes, establishing the first mechanism that could decide the destiny of an MVE – fusion with either a lysosome or the PM. It also supports the notion that the first subunits of the ESCRT mechanism could be used in secretory and degrading MVEs; it would therefore be interesting to know whether ISGylation of TSG101 determines recruitment of either the conventional ESCRT machinery for degradation or ALIX for secretion. Tetraspanin 6 (Tspan6) has also recently been involved in the regulation of this balance between lysosomal degradation and exosomal secretion. Overexpression of Tspan6 slows down lysosomal degradation of APP-CTF and enhances its secretion on exosomes probably by recruiting syntaxin [84].

How can a particular mechanism of sorting act on the motility and polarized transport controlling the fate and function of a subpopulation of MVEs? Distinct subsets of endosomes destined, respectively, for degradation and secretion could be exclusively associated with ESCRT-dependent and -independent sorting mechanisms. Alternatively, ESCRT-dependent and -independent sorting pathways could act together on the same MVE, and the abundance of a given cargo protein or its PTM will favour the recruitment of one sorting mechanism over another, and dictate the destiny of the MVE. Finally, as observed in specialized cells such as melanocytes, ESCRT-independent and -dependent sorting could act sequentially, with the former acting first whereas cargo proteins destined for the latter are sequestered into a clathrin coat at the limiting membrane. Only early MVEs could then be targeted for secretion. The cell type and its maturation state would be important, as shown for dendritic cells that use different sorting mechanisms according to their maturation stage and reticulocytes that use different mechanisms for exosome secretion during their maturation into erythrocytes. Finally, the only feature that has allowed discrimination of subpopulations of MVEs with apparent distinct destinies is their cholesterol content [85]. This seems to be of prime importance for better understanding how sorting mechanisms could modulate the concentration and localization of this lipid at and within MVEs, especially given the role of cholesterol in endosomal motility [86].

In addition to lysosomes, MVEs can fuse with autophagosomes, forming an amphisome [87]. This organelle then fuses with a lysosome for degradation of its content, in a process called macroautophagy. Despite a lack of data on the potential link between the macroautophagy signalling pathway and exosomes, there may be a similar balance between exosome secretion and autophagy which would depend on external stimuli such as starvation (see Figure 1d'). In support of this hypothesis, it has been shown that the prion protein (PrP) can promote exosome secretion by inhibiting autophagosome formation through interaction with caveolin 1, a suppressor of autophagosome formation [88]. Moreover, inhibition of the kinase PIKfyve, which is also involved in autophagy, increases secretion of a subpopulation of 'autophagic' EVs enriched with autophagic proteins, but not with 'classic exosomal markers', again suggesting that impairment of autophagy can be balanced by secretion [89]. Interestingly different autophagy proteins have recently been shown to regulate exosome secretion, such as the ATG12–ATG3 complex which controls both autophagy and exosome secretion via interaction with ALIX [90], or ATG5 which induces sequestration of a subunit of  $V_1V_0$ -ATPase in ILV, inhibiting in this way ATPase activity, and promotes MVE acidification and exosome secretion [37]. In addition, the tetraspanin CD63 has recently been proposed to coordinate both autophagic and endosomal processes and to regulate exosomal secretion of EBV-encoded LMP1 [91].

Finally, it is noteworthy that not only MVEs but also lysosomes and autolysosomes can secrete their content into the extracellular environment, including any remaining ILVs. Even though these pathways could use different mechanisms, it is difficult to distinguish their respective contributions to exosome or extracellular vesicle biogenesis, especially as they can all contribute to the maintenance of cell homeostasis and probably share common signalling pathways.

## Targeting MVEs to the PM for secretion

Regardless of their fate, MVEs have to be transported before fusing with lysosomes or the PM. This step provides a final checkpoint for exosome biogenesis (see Figure 1e). In general, intracellular transport of organelles requires association with the cytoskeleton, together with the action of molecular motors and small GTPases, and their fusion involves soluble NSF attachment protein receptor (SNARE) proteins [92,93]. In this emerging topic, the precise involvement of these mechanisms in exosome secretion would benefit from further investigations.

Several Rab proteins have been involved in exosome secretion. Use of a dominant negative mutant of RAB11, a GTPase mostly known for its role in recycling cargo proteins from EEs to the PM, decreases secretion of TfR and HSC70 in exosomes from the leukaemia cell line K562 [94], whereas in *Drosophila* S2 cells, RAB11 depletion inhibits secretion of EVs carrying Wingless [95] or Evi [96] types. In addition, depletion of RAB11 or RAB35 in retinal epithelial cells decreases exosomal secretion of flotillin and anthrax toxin [97]. Other studies confirmed the involvement of RAB35 in exosome secretion from primary oligodendrocytes [98] or oligodendroglial cell lines, where RAB35 seems to have a role in the docking and tethering of MVEs to the PM [99]. An shRNA-based screen in HeLa cells expressing MHC-II molecules showed that silencing of RAB2B, RAB5A, RAB9A, RAB27A and RAB27B decreased CD63, CD81 and MHC-II secretion in exosomes [7]. In more detail, the same study showed the involvement of RAB27A/B in the docking of MVBs to the PM and several subsequent studies have confirmed, in different cell lines, the reduction in exosome secretion after RAB27A silencing; this strategy is now commonly used as a way of modulating exosome secretion [45,100–102]. Finally, RAB7 has been involved in secretion of syntenin–ALIX exosomes by MCF-7 cells [12], whereas its depletion does not affect exosome secretion in HeLa cells [7]. The fact that both early and late endosomal Rab proteins have a role in exosome secretion suggests that, according to cell type, different subpopulations of MVEs originating either from early or recycling endosomes or from late endosomes are used to release ILVs as exosomes. Alternatively, the involvement of so many Rab proteins acting on the various compartments that cross the endosomal pathway would confirm the interdependence of the different steps controlling the endosomal pathway in the regulation of exosome biogenesis.

The importance of the cytoskeleton is supported by the specific location of exosome release at the PM, as in the case of immunological synapses in antigen-presenting cells [69,103] or invadopodia [45] in cancer cells. This polarized secretion suggests that cells would not release exosomes over their entire surface but use polarized transport to target secretion towards a precise location at the PM. The microtubule network, which contributes to the overall intracellular organization and the polarization of intracellular compartments, would be of prime importance to transport MVEs, in coordination with molecular motors, to the site of secretion. RAB7 is a key regulator of MVE motility because it can interact with the dynein motor through its effector Rab-interacting lysosomal protein (RILP), favouring the transport of late endosomes towards the microtubule organizing centre (MTOC) [104] where lysosomes localize. Such interaction is regulated by the ubiquitinated state of RAB7 [33] and the presence of cholesterol in the limiting membrane of MVEs [86]. This suggests that the composition of the limiting membranes of MVEs may modulate their

destiny by acting on the motility of MVEs. Further investigation of these mechanisms would also shed new light on the relationship between the destiny of MVEs and the associated endosomal sorting mechanisms, because the latter would modify the composition of the limiting membrane of MVEs differently through the generation of ILV subpopulations. Actin cytoskeleton would also affect exosome release, because it is required for the docking and fusion of MVEs with the PM. The actin regulatory protein cortactin has been shown to promote exosome secretion by regulating both trafficking and docking of MVEs to the PM together with the small Rab GTPase, RAB27A and coronin1b [105]. At this stage, the mechanisms involved in docking and fusion of MVEs with the PM are not completely known, although some Rab GTPases, SNARE proteins and synaptogamin family members participating in this process have been identified. As previously mentioned, RAB27A and RAB27B (together with their respective effectors, synaptotagmin-like protein 4 and exophilin 5) act in the docking of MVEs to the PM in order to promote their fusion [7] and this mechanism requires the rearrangement of the actin cytoskeleton [105]. The SNARE protein VAMP7 has been involved in exosome secretion in the leukaemia cell line K562 [106] and expression of a form of VAMP7, which inhibits SNARE complex formation, decreases exosome secretion. Another SNARE protein, YKT6, has been shown to be required for exosome release in HEK239 cells [36] and A549 lung cancer cells [107]. In *C. elegans*, the Ras-related GTPase homologue Ral-1 is involved in MVE biogenesis and their fusion with the PM together with the SNARE protein, syntaxin 5 [108]. Recently, phosphorylated SNAP23 has been shown to enable exosome release [66,109]. Of note, new tools for direct visualization of exosome secretion in live cells, such as CD63-pHluorin [66,110], open new avenues to map and understand the release of exosomes in a single cell. In this context, the release of  $Ca^{2+}$  into the cytoplasm seems to have a different requirement for the activation of the SNARE complex involved in MVE-PM fusion, revealing a cell type-specific contribution of this known regulator of exosome secretion [66,111-113]. This last checkpoint determines exosome functions, because it will orient exosome secretion towards either the correct cellular partner of a synapse [69] or the correct body fluids or biological barrier for reaching its final destination.

## Exosome secretion: constitutive or regulated process?

The presence of exosomes in cell culture media from cells under basal conditions suggests that exosome secretion can be a constitutive process but also a side effect of cell death. Nevertheless, external or internal stimuli received by the producing cells can modulate basal exosome biogenesis and/or secretion. As mentioned earlier, several studies have shown that the release of  $Ca^{2+}$  in the cytoplasm regulates exosome secretion in specific cell types [66,111-113]. Moreover, pathological conditions such as inflammation or cancers can also modulate exosome secretion or their composition. In this case, external stimuli can induce specific intracellular signalling which would then modulate exosome biogenesis and/or secretion. In the context of inflammation, stimulation of intestinal cells with IFN- $\gamma$  up-regulates the expression of MHC-II and its secretion on exosomes [8]. In addition exosomal release of MHC-II by dendritic cells is stimulated by their interaction with CD4<sup>+</sup> T-cells [69], showing the importance of intercellular communication in modulation of exosome release. In the context of cancer, histamine, a ligand of GPCRs, has recently been shown to induce exosome secretion, visualized using a CD63-pHluorin tool, via a signalling pathway that involves protein kinase C [66]. Furthermore, mutations in KRAS, which frequently occur in cancer, modulate release of Ago2 and specific miRNAs in exosomes through the MEK-ERK pathway [114], whereas EVs from breast cancer cells with HER2 amplification were enriched in proteins that can promote malignant transformation [115]. Recently the cytoplasmic tyrosine kinase, SRC, has also been shown to stimulate secretion of syndecan-syntenin-positive exosomes with pro-migratory capacity [116]. Finally, chemotherapy can be seen as an external stimulus that modulates exosome secretion by cancer cells. These exosomes, once secreted, mediate resistance to chemotherapy, mostly by two mechanisms. First, exosomes can present a way for cells to remove drugs from their cytosol, as shown in the case of cisplatin-resistant ovarian carcinoma cells [117]. Interestingly, these cells seem to have an abnormal lysosomal compartment compared with cisplatin-sensitive cells, suggesting that exosomal secretion of cisplatin could compensate for lysosomal impairment. Second, exosomes secreted by resistant cells can transfer active molecules such as miRNAs to sensitive cells, so inducing resistance [118]. It appears that each checkpoint that controls exosome biogenesis is a potential target for external stimuli.

## Conclusion

The multiple checkpoints that have been described in the present review as potential regulators of the biogenesis of exosomes are all subjected to regulatory pathways that can be stimulated or inhibited under physiological or pathological conditions [119]. This implies that exosome biogenesis and secretion are probably part of an inducible pathway that is modulated according to which stimuli are received by the producing cells rather than a constitutive pathway. Once released into extracellular space, exosomes can reach body fluids, such as blood, and travel through the whole

body, supporting the targeting of destinations that are far from their site of production. Alternatively, exosomes can remain closely associated with the cells that produced them, notably through a mechanism involving the presence of tetherin [76]. The mechanisms involved in the biogenesis of exosomes, through the modulation of their composition, will then be critical in specifying their uptake by recipient cell types or regulating their destination, as shown *in vivo* for tumour exosomes which can reach and stop in different organs according to the set of integrins that they contain [120]. Once at a destination in recipient cells, exosomes are endocytosed and follow the endosomal pathway to provide signaling or trophic support. The same intracellular mechanisms than those described in producing cells to generate exosomes are then involved in the regulation of the fate and functions of exosomes in recipient cells, strengthening the importance of cell biology to better understand the relevance of extracellular vesicle mediated communication.

## Summary

- Biogenesis of exosomes is regulated by various checkpoints throughout the endosomal pathway.
- A first checkpoint regulates the targeting of exosomal cargo proteins from the plasma membrane to the multivesicular endosome.
- A second checkpoint allows sorting of cargo proteins to intraluminal vesicles of multivesicular endosomes via several sorting mechanisms, which also influence the compartment's fate.
- A third checkpoint controls the balance between the degradation of multivesicular endosomes and their targeting to the plasma membrane and secretion.
- The intracellular pathways involved in exosome biogenesis determine their function because they regulate composition, specific release and fate of exosomes.

## Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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## Author Contribution

All authors contributed equally to all aspects of the article (researching data for article, figures substantial contribution to discussion of content, writing, and review/editing of manuscript before submission).

## Abbreviations

ApoE, apolipoprotein E; APP, amyloid precursor protein; Arf6, ADP-ribosylation factor 6; CI, clathrin-independent endocytosis; DAG, diacylglycerol; EBV, Epstein-Barr virus; EE, early endosome; EGFR, epidermal growth factor receptor; ESCRT, endosomal sorting complex required for transport; EV, extracellular vesicle; GPCR, G-protein-coupled receptor; GPI, glycosyl phosphatidylinositol; IFN- $\gamma$ , interferon- $\gamma$ ; ILV, intraluminal vesicle; LMP1, EBV-encoded latent membrane protein 1; MVE, multivesicular endosome; PI<sub>3</sub>P, phosphatidylinositol 3-phosphate; PI(4,5)P<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PM, plasma membrane; PMEL, premelanosome protein; PTM, post-translational modification; RE, recycling endosome; SDC-CTF, syndecan C-terminal fragment; SNARE, soluble NSF attachment protein receptor.

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# EV-TRACK: transparent reporting and centralizing knowledge in extracellular vesicle research

EV-TRACK Consortium\*

We argue that the field of extracellular vesicle (EV) biology needs more transparent reporting to facilitate interpretation and replication of experiments. To achieve this, we describe EV-TRACK, a crowdsourcing knowledgebase (<http://evtrack.org>) that centralizes EV biology and methodology with the goal of stimulating authors, reviewers, editors and funders to put experimental guidelines into practice.

EVs have emerged as having important (patho)physiological roles, and as such they have been the focus of intense study over the past decade<sup>1–4</sup>. Despite substantial progress, the complexity and challenges associated with EV research remain considerable<sup>5</sup>. EVs released from different cell types (and even from a single cell type) are heterogeneous in size and in protein, nucleic acid and lipid content<sup>6–9</sup>. The isolation of EV populations is frequently complicated by the presence of contaminants with comparable features<sup>10,11</sup>. Different isolation methods enrich for single or multiple EV subtypes with diverse composition and variable purity, thus identifying method-dependent EV content and function<sup>6–11</sup>. Each detection and characterization method has its own accuracy and precision in measuring EVs<sup>12,13</sup>. Still, functional studies on EVs vastly outnumber those focusing on EV biology and methodology (**Supplementary Fig. 1**).

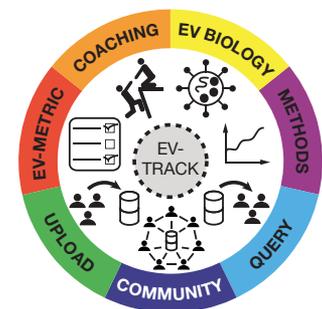
In EV research, as in any field, the implementation of different methods requires validated controls and adequate reporting of experimental parameters. Failure to follow these principles can result in data that are difficult to interpret and reproduce<sup>14</sup>. Similar to minimal information checklists in other fields<sup>15–17</sup>, the International Society for Extracellular Vesicles (ISEV) introduced minimal informations for studies on EVs (MISEV) guidelines<sup>18,19</sup>. Nevertheless, EV research

is unfolding at a rate that impedes widespread adoption of these guidelines.

We convened an international consortium to develop the EV-TRACK knowledgebase, which records experimental parameters of EV-related studies. EV-TRACK implements a bottom-up community consensus approach and encourages researchers to upload published and unpublished experiments and provide feedback. It is a unique resource that was developed to (i) create an informed dialog among researchers about relevant experimental parameters, (ii) improve the rigor and interpretation of experiments studying EVs, and (iii) record the evolution of EV research. The final goal of the EV-TRACK consortium is to facilitate standardization of EV research through increased systematic reporting on EV biology and methodology.

## Creation of the EV-TRACK knowledgebase

Currently, the EV-TRACK knowledgebase comprises methodological specifications of 1,226 EV-related articles published in 2010–2015 (**Supplementary Fig. 2**). Publications that included multiple sample types or isolation methods were separated into multiple entries, resulting in 1,742 experiments (**Supplementary Methods**). For each experiment, we completed a checklist of 115 parameters—based partially on the MISEV guidelines<sup>19</sup>—relating to sample type, preanalytical variables, isolation protocol and characterization method (**Supplementary Table 1**). Data were curated before inclusion in the knowledgebase, which can be accessed freely at <http://evtrack.org>.



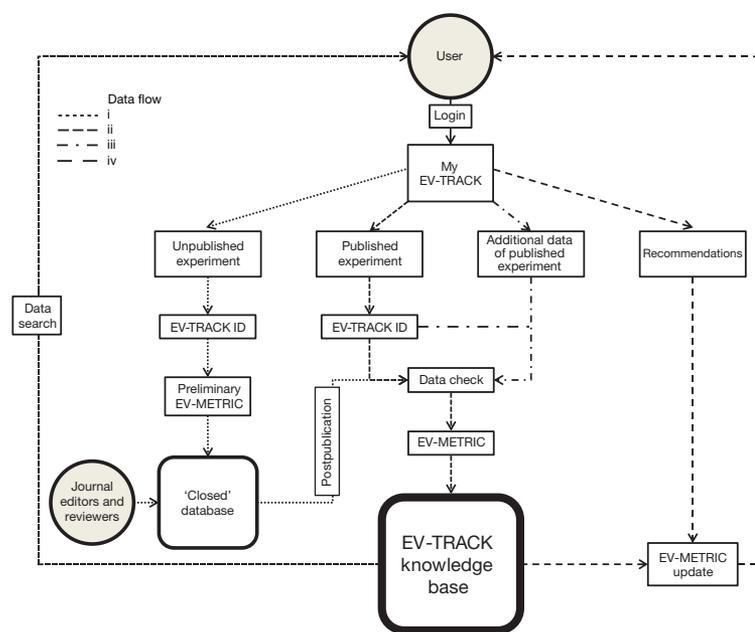
**Figure 1** | The seven enabling features of the EV-TRACK platform.

## EV research practices

To assess current practices in EV experiments, we performed an in-depth analysis of the data in the EV-TRACK knowledgebase. This revealed widespread heterogeneity in EV isolation methods and inconsistent implementation and reporting of important experimental parameters, including isolation methods and characterization of EV size, composition and purity (**Supplementary Fig. 3**).

The 1,742 experiments that are recorded in EV-TRACK report 190 unique isolation methods and 1,038 unique protocols to retrieve EVs from biofluids (**Supplementary Tables 2 and 3**). Differential ultracentrifugation (dUC) is the most popular method (45% of all experiments), but with variable parameters selected by researchers, even for experiments handling a similar sample type. For cell culture supernatant ( $n = 813$  experiments using dUC), 218 unique combinations of centrifugation steps and final pelleting times are recorded, along with a

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**Figure 2** | Implementation of the EV-TRACK knowledgebase. A flowchart demonstrating four different data flows available to registered EV-TRACK users. (i) Study authors are able to upload prepublication data. Upon upload, an EV-TRACK ID is assigned, and preliminary EV-METRIC is calculated based on the specified parameters. Upon publication, the data submitted in EV-TRACK are curated by the EV-TRACK administrators, the final EV-METRIC is calculated, and the experiment(s) is(are) included and searchable in the public knowledgebase. (ii) Data of published experiments that are submitted to EV-TRACK can immediately be curated and included in the main database. (iii) Unpublished data of an experiment can be added to increase reporting transparency. (iv) EV-TRACK users can provide recommendations which will be considered together with data from the knowledgebase to update EV-TRACK and EV-METRIC.

wide variety of rotor types ( $n = 43$ ). While the ratio of  $k$ -factor to pelleting time should be constant to pellet objects with a similar sedimentation coefficient<sup>20</sup>, this is not the case (**Supplementary Fig. 4**). Overall, nearly all dUC experiments report on  $g$ -forces and durations of different UC steps (96%); only 28% of experiments report on rotor type. In just over 18% of experiments, a density gradient was implemented to obtain or at least validate results, with 30% and 60% of these reporting rotor type or EV density, respectively. Use of density gradients decreases over time, for the period we analyzed, and is accompanied by increased implementation of commercial isolation methods (**Supplementary Fig. 5**). Alternative but less frequently recorded isolation methods include immunoaffinity capture<sup>6,21</sup> (2.2%) and size-exclusion chromatography<sup>22</sup> (5.6%).

17% of experiments provide no characterization of EVs; in 29% and 39% of experiments characterization was limited to protein or particle analysis, respectively. Unbiased characterization of EV proteins by proteomics is performed in 16% of experiments. In 55% of experiments only an antibody-based assay was included to

detect EV proteins. 21%, 25%, 26% and 29% of these experiments report the presence of 1, 2, 3 or >3 proteins, respectively. Tree maps reveal CD63, CD9, CD81, PDCD61P (Alix) and TSG101 as the most commonly evaluated EV-enriched proteins (**Supplementary Fig. 6**). Evaluation of contaminating proteins in EV preparations is done in 17% of experiments and typically limited to organellar proteins. When antibodies are used, clone or catalog number and dilution factor are reported in 14% of experiments. Preparation of lysis buffers is detailed in 29% of experiments. 18% of experiments include both qualitative (electron microscopy (EM) or atomic force microscopy (AFM)) and quantitative (EM, nanoparticle-tracking analysis (NTA), dynamic light scattering (DLS), tunable resistive pulse sensing (TRPS), high-resolution flow cytometry (hrFC)) particle analysis. Transmission electron microscopy (TEM), NTA and DLS are the most used particle analysis methods (used in 41%, 17% and 6% of experiments, respectively). Immuno-EM is performed in 10% of experiments, with CD63 as the most frequently evaluated protein (36%). EV size, as measured by EM image

analysis, is reported in 3% of experiments. Alternative but less frequently recorded particle analysis methods are cryo-EM, scanning-EM, TRPS, AFM and hrFC (used in a total of 11% of the recorded experiments).

The heterogeneity revealed by this analysis demonstrates the need for reporting guidelines to improve evaluation and reproducibility of EV experiments. We were motivated by these findings to develop the EV-METRIC, described below and in **Box 1, Figure 3**, to improve the completeness of methodological reporting of EV-based experiments.

### Using the EV-TRACK platform

The EV-TRACK platform comprises seven enabling features to assist researchers (**Fig. 1**).

**Upload.** Researchers can upload EV experiments through an online interface. As part of each upload, experimental parameters including sample type, preanalytical variables, isolation protocol and characterization method are recorded. Each annotated experiment receives a unique identifier, the EV-TRACK ID (**Fig. 2**). Unpublished EV experiments are contained in a 'closed' section of the database, only accessible to the study authors, editors and reviewers. After acceptance, study authors can modify the submission to reflect what is reported in the peer-reviewed manuscript, and they can add the PubMed identifier.

The annotation of experiments will be publicly accessible after curation by the EV-TRACK administrators. Study authors are notified when experiments are uploaded to EV-TRACK. They can then add more detailed descriptions of experiments reported in the original publication (e.g., UC specifics, antibody details, EM images). These postpublication adaptations will be flagged to distinguish them from the peer-reviewed parameters. To ensure data integrity, modifications to any entry can be requested for consideration by the EV-TRACK administrators based on what is reported in the corresponding peer-reviewed article.

**EV-METRIC.** EV experiments get assigned an EV-METRIC based on the reported experimental parameters (**Box 1, Fig. 3**). This metric assesses whether enough information has been provided to interpret and reproduce the experiment. It consists of nine components that stipulate validation experiments and experimental parameters

that our consortium argued to be indispensable for unambiguous interpretation and independent replication of EV experiments. Some of the challenges are not unique to the EV field and have been discussed previously<sup>14,23</sup>. Other components (sections 2 and 3 on protein and particle analysis) are included in the MISEV guidelines<sup>19</sup>.

The EV-METRIC reflects reporting in an experiment according to the version of the

metric at the time of uploading. However, the metric is necessarily dynamic, meaning that its defining components can change as the field evolves. We have developed it primarily to pave the way toward standardized EV research; it is not our intention to impede studies on EVs that are rare or poorly described. When submitting data to EV-TRACK, authors have the option to indicate why one or more components of

the EV-METRIC were not adhered to, which will be displayed as a note together with the EV-METRIC summary.

**Query.** Users can query the database for articles using a range of search parameters. The query results list is accompanied by an overview of the most common EV isolation protocols and EV-enriched proteins. For each experiment, the EV-METRIC, its percentile across other annotated experiments

## BOX 1 THE EV-METRIC

We extracted nine relevant experimental parameters that we condensed into a single metric, the EV-METRIC. It represents a checklist to assess the completeness of reporting of generic and method-specific information necessary to interpret and reproduce the experiment (**Supplementary Table 5**). The EV-METRIC describes the type of information that should be provided for EV experiments in three sections.

### Section 1: isolation method

#### A. Density gradient, at least as validation of results attributed to EVs

#### B. EV density

#### C. Ultracentrifugation specifics: *g*-forces, duration, rotor type (conditional)

Density gradient centrifugation separates EVs and non-EV structures based on differences in buoyancy, making it currently the only isolation method that eliminates the majority of contaminants<sup>6,10,11,21,30–33</sup>. It should at least be implemented to validate an EV experiment (i.e., to confirm presence of the molecule(s) of interest and/or attributed functions in the EV fraction of the gradient). Subsequent reporting of the equilibrium density of gradient fraction(s) containing the EVs allows identification and comparison of different EV subtypes<sup>6–8</sup>. In case EVs are isolated through differential ultracentrifugation, rotor type, applied *g*-forces and duration of centrifugation steps should be specified to allow interpretation and replication of the protocol (**Supplementary Table 5**)<sup>20,34</sup>.

### Section 2: protein analysis

#### A. Analysis of three or more EV-enriched proteins

#### B. Assessment of a non-EV-enriched protein

#### C. Antibody specifics and dilution (conditional)

#### D. Lysis buffer composition (conditional)

In addition to EV density, recent evidence shows that combinations of differentially enriched proteins can discern EV subtypes<sup>8,10,26,29,35</sup>. Reporting on the presence of multiple (three or more) proteins in the EVs of interest, whether via biochemical assays or omics approaches, is therefore included in the EV-METRIC. Given the current lack of a consensus on proteins that can be considered EV subtype ‘markers’, we did not include a priori restrictions on proteins that should be evaluated. Reporting on the absence of one or more proteins in EV preparations is a way of evaluating contamination by non-EV entities<sup>10,18</sup>. In case antibody-based assays are used, reporting their details (reference, provider, clone, dilution) is a general requirement in biochemical research<sup>23</sup>. If EVs are lysed for protein analysis, reporting on the composition of the lysis buffer and conditions of the lysate preparation allows interpretation of western blot bands.

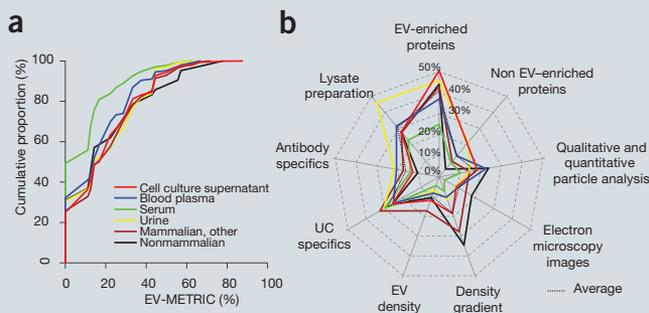
### Section 3: particle analysis

#### A. Quantitative and qualitative analysis

#### B. Widefield and close-up electron microscopy image

Close-up and widefield images obtained by electron microscopy or similar methods (e.g., AFM) allow evaluation of both EV morphology and the presence of non-EV structures<sup>36–38</sup>. Particle yield can be determined by quantitative analysis of EM pictures, bulk (DLS) or single-particle methods (e.g., NTA, TRPS, hrFC)<sup>13,37</sup>.

Since most of the EV-METRIC’s experimental parameters are poorly reported as identified by EV-TRACK data mining, experiments generally had low EV-METRICs (**Fig. 3** and **Supplementary Table 6**). A cumulative plot reveals that less than 6% of experiments obtain an EV-METRIC above 50% (**Fig. 3a**). Moreover, almost 30% of experiments fail to adhere to a single EV-METRIC component. The average EV-METRIC across all biofluids is 20%, and the maximum lies at 88%. Experiments on serum-derived EVs generally have the lowest metric, with the average being 11% ( $P < 0.001$ , Kolmogorov–Smirnov test). A spider chart (**Fig. 3b**) shows that some of the EV-METRIC parameters are reported relatively more often (EV-enriched proteins, UC specifics, lysis buffer composition) than others (non-EV-enriched proteins, EV density, EM images, antibody specifics).



**Figure 3** | Using the EV-METRIC to evaluate transparent reporting in EV research. **(a)** Cumulative frequency plots showing the cumulative proportion of experiments achieving a certain EV-METRIC. **(b)** Spider chart representing the percentage of experiments that adhere to each of the respective EV-METRIC parameters for indicated biofluids. Color codes for biofluids are identical in **a** and **b**, with the addition of the average as a black dotted line in **b**.

and raw annotation data can be consulted. EV-TRACK querying allows EV researchers to find and compare specific information of published experiments with their own unpublished data. All published experiments are accessible without the need for registration.

**Coaching.** By facilitating the search and comparison of EV-related publications, EV-TRACK familiarizes EV researchers with relevant experimental parameters.

**Methods.** The EV field is rapidly evolving with new methods to isolate and characterize EVs. Community annotation enables identification of these methods and helps to monitor if and when experimental guidelines are required.

**EV biology.** Beyond experimental parameters, EV-TRACK systematically catalogs biochemical and physical characteristics of EVs. This will provide insights into the basics of EV biology, such as the identification of EV subsets and optimal protocols to isolate them.

**Community.** EV-TRACK aims to increase standardized reporting of experimental parameters using a community consensus approach. Registered EV-TRACK users will be involved in future decision making on EV-TRACK and its EV-METRIC by submitting their recommendations.

### Discussion and future perspectives

Our analyses reveal that a large number of publications on EVs contain insufficient information for unambiguous interpretation or replication of experiments. We established the crowdsourcing EV-TRACK platform as a next step toward increasing experimental rigor, enhancing biological knowledge, and creating timely and mature minimal information checklists.

EV-TRACK data mining identified a need to guide EV researchers in specific aspects of EV isolation and characterization, which led us to develop the EV-METRIC. Although the EV-METRIC's experimental parameters are currently poorly reported, we believe that the application of this system will not impose an unrealistic burden on researchers. It is meant as an incentive to report parameters that hitherto often remained unreported. In 81% of examined experiments, an increase of the EV-METRIC would already have been achieved by increased reporting, without additional analyses (**Supplementary Table 4**).

The EV-METRIC is applicable to the majority of EV experiments in literature.

However, we recognize that it can be challenging to adhere to certain components of the metric, for example if samples are difficult to obtain or limited in volume. The EV-METRIC is not meant to restrict the study of EVs in such cases, and EV-TRACK allows the transparent discussion of any issues that might render the EV-METRIC less suitable. If possible, we propose for these studies that researchers validate the most appropriate method on a test sample.

The diversity and constant evolution of the EV research field, including constant methods development, requires the EV-METRIC to be dynamic. Systematic cataloguing of isolation and characterization methods, experimental parameters and information on EV subtypes by EV-TRACK will fuel future iterations of the metric. For example, combinations of isolation methods are likely required to explore the full range of EV subtypes with immunoaffinity capture and size-exclusion chromatography to complement the use of density gradients<sup>6,22</sup>. Technological progress will allow isolation-independent identification and characterization of EVs in different biofluids (e.g., hrFC<sup>24</sup> or microfluidics devices<sup>25,26</sup>). The use of quantitative technologies such as NTA, TRPS and hrFC will drive the inclusion of additional method-specific guidelines in the EV-METRIC<sup>12</sup>. We hope that future work will establish guidelines for analysis of the RNA content of EVs, since this is highly affected by the purity of an EV preparation<sup>10,27,28</sup>, as well as for EV treatments in cell culture and/or animal models<sup>29</sup>.

The widespread implementation of EV-TRACK and EV-METRIC will depend on the scientific community at large. We recommend that funding organizations, editors and editorial board members encourage reviewers to implement EV-TRACK and EV-METRIC to adequately identify potential limitations in EV-related grant applications and manuscripts. As such, the EV-TRACK consortium aims to increase experimental rigor in order to help the EV field to mature and reach its full potential.

**Data availability statement.** All data that were collected during the course of this study and that support its findings are available online at <http://evtrack.org>.

*Note: Any Supplementary Information and Source Data files are available in the online version of the paper.*

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J.V.D., P.M., O.D.W., J.V. and A.H. designed the study and analyzed and interpreted the data. J.V.D., P.M., P.A., G.B., J.G., B.G., A.F.H., S.M., E.N.M.N.-t.H., L.O., M.W.P., S.S., J.V.S., C.T., G.V.N., M.W., K.W.W., O.D.W., J.V. and A.H. discussed and prepared the manuscript. J.V.D., P.M., J.A., O.D.W., J.V. and A.H. developed the online tool. All authors annotated data and approved of the final manuscript.

### COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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