The Tetraspanin Web in Exosome Biogenesis

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1. Introduction [ϕ]

The term "exosome" was originally used to describe small vesicles released during reticulocytes maturation (Johnstone *et al.* 1987), and may function in shedding redundant or useless plasma membrane proteins, such as transferrin receptor (Johnstone *et al.* 1984) or acetylcholine esterase (Johnstone *et al.* 1987). Exosomes should not be confused with the ribonuclease complex that has also been named exosome (Mitchell *et al.* 1997). As do all lipid vesicles, exosomes float on sucrose gradients and their density ranges from 1.13 g/ml to 1.19 g/ml. Exosomes present a characteristic "cup shaped" morphology (Thery *et al.* 2002; Chaput *et al.* 2004). Later study shows that B lymphocytes and immature dendritic cells (DCs), as well as other antigen presenting cells (APCs), are able to secret exosomes that are loaded with major histocompatibility complex (MHC) molecules (Raposo *et al.* 1996; Zitvogel *et al.* 1998).

1.1 Origin and Biogenesis of Exosomes

Exosomes are small bilayer vesicles (diameter: 50-100 nm) secreted by hematopoietic and epithelial cells (Laulagnier *et al.* 2004), which are formed by inwardly budding of late endosome (Figure 1). Cells absorb material outside their cell membranes by pinocytosis, receptor-mediated endocytosis or phagocytosis. In metazoan cells, endocytosis is a source of nutrients, growth factors, pathogenic substances, *etc* (Surmacz *et al.* 2003). Endosomes are formed by endocytosis and maturate by trafficking inside the cell (van Deurs *et al.* 1993). Late endosomes, referred to as multivesicular bodies (MVBs), have numerous internal vesicles, which are precursors of exosomes and generated by reverse budding (Peterson *et al.* 1998).

[ϕ] Abbreviations used: DCs, dendritic cells; APCs, antigen presenting cells; MHC, major histocompatibility complex; MVBs, multivesicular bodies; MCs, mast cells; TEMs, tetraspanin enriched microdomains; SM, sphingomyelin; SMase, sphingomyelinase; LCLM, live-cell light microscopy; IEM, immunoelectron microscopy; FACS, fluorescence-activated cell sorting; MS, mass spectrometry; IP, immunoprecipitate.

Transporting MVBs towards the cell periphery depends on microtube and kinesin's function (Wubbolts *et al.* 1999). MVBs fuse with the plasma membrane, and release internal vesicles into the extra-cellular milieu as exosomes, which is accompanied by increased intracellular [Ca^{2+}] (Savina *et al.* 2003). Synaptotagmin, Rab and SNARE proteins are thought to function in MVBs membrane targeting process (Reddy *et al.* 2001; Savina *et al.* 2002).

Biogenesis of exosomes is divided into two sequential steps: the first step involves the selection at the endosome membrane; the second step happens during the inwardly budding (Stoorvogel *et al.* 2002). Although the precise role is not clear yet, PI(3)P and PI(3,5)P_2 are required for the biogenesis in MVBs (Odorizzi *et al.* 1998; Futter *et al.* 2001). Protein sorting during exosome biogenesis appears to be mediated by several different mechanisms including direct binding, fatty acylation, aggregation and monoubiquitylation (Gould *et al.* 2003).

1.2 Composition of Exosomes

Specific proteins and lipids are highly concentrated in exosomes. Exosomes have a unique protein composition, and all proteins that have been identified in exosomes are only found in the cytosol or the plasma membrane, not other organelles. They include chaperones such as Hsc73 and Hsc90, integrins, subunits of trimeric G proteins, Tsg101, cytoskeletal proteins such as actin, profilin, cofilin and tubulin, and tetraspanins (CD9, CD63, CD81, CD82 and CD151). B lymphocytes, DCs, mast cells (MCs) and intestinal epithelial cells secret exosomes enriched in MHC class I and II molecules, whereas exosomes from platelets and cytotoxic T cells contain von Willebrand factors (Heijnen *et al.* 1999) and granzymes (Peters *et al.* 1991).

Tetraspanins, the most abundant protein family found in exosomes (Chaput *et al.* 2004), are a distinct family of proteins, containing four transmembrane domains, a small extracellular

motif (EC1), a large extracellular loop (EC2), a small inner domain and a short cytoplasmic tail (Figure 2) (Hemler 2003; Stipp *et al.* 2003). EC1 is essential for optimal expression of EC2. EC2 has a constant putative dimerization region and a variable protein-protein interaction region. Intra- and inter-molecular interactions between transmembrane domains are crucial to stabilize tetraspanin in the membrane. Divergent C-terminal tails provide specific links to cytoplasmic factors and function distinctly. One fundamental role of tetraspanins appears to organize other proteins into a multimolecular tetraspanin enriched microdomains (TEMs), in which palmitoylated tetraspanins show robust, specific oligomerization. The tetraspanin web, defined as the extended network of tetraspanins interactions in the membrane (Boucheix and Rubinstein 2001), is further formed by increased interaction between different TEMs and between tetraspanins and many other membrane proteins. In addition, the association with lipids, such as cholesterol and sphingomyelin (SM), might contribute to the assembly of the tetraspanin web. CD9, a tetraspanin family member, is soluble in chloroform/methanol, interacts with gangliosides, and classifiable as "proteolipid" (Kawakami *et al.* 2002), which gives a hint that tetraspanins work together with lipids. Since tetraspanins are known to form protein complexes and function in highly ordered raft-like domains (Claas *et al.* 2001), they may play a role in exosome biogenesis. In addition, a highly specialized tetraspanin complex has the diameter about 16-nm diameter (Liang *et al.* 2001), which is very convenient to be a "pieces" of a exosome (50-100 nm).

The lipid composition in exosomes is different from either the plasma membrane or early endosomes. Lysobisphosphatidic acid is efficiently incorporated into the MVBs (Kobayashi *et al.* 1999), and is thought to regulate intracellular cholesterol transport by acting as a collection and distribution device (Kobayashi *et al.* 1999; Holtta-Vuori *et al.* 2000; Lebrand *et al.* 2002).

Cholesterol and SM are highly enriched in purified exosomes. On the surface of exosomes from platelets and DCs, phosphoserine is also present, though at low level (Chaput *et al.* 2004).

1.3 Functions of Exosomes

Since exosomes are membrane coated vesicles, whether they fuse with target cells or not, their function is related to which membrane and membrane proteins they are transferring. During reticulocytes maturation, exosomes are secreted to discard membrane proteins, so exosomes are treated as a possible alternative to lysosomal degradation in early studies. According to their unique composition, exosomes should play more diverse and important roles in numerous physiological events. Today's working hypothesis in the field is that exosomes function in T lymphocyte activation, immunological tolerance, and pattern formation in early *Drosophila* development, and they are implicated in cancer immunotherapy as well.

Exosomes from APCs, associated with MHC molecules, directly stimulate T cell response, and have important roles in intercellular communication during immune responses (Raposo *et al.* 1996; Zitvogel *et al.* 1998; Wolfers *et al.* 2001; Skokos *et al.* 2003). Raposo *et al.* showed that both the limiting membrane and the internal vesicles of MVBs harbored newly synthesized MHC class II, and the limiting membrane fused directly with the plasma membrane, resulting in release of internal MHC class II-containing vesicles. These secreted exosomes derived from both human and murine B lymphocytes could induce antigen-specific MHC class II-restricted T cell responses. Zitvogel *et al.* showed that tumor peptide-pulsed DC-derived exosomes could prime specific cytotoxic T lymphocytes in vivo and suppress growth of established murine tumors in a T-cell-dependent manner. The authors thought that DC-derived exosomes might transfer MHC-peptide complex between different DCs, thereby increase the number of

peptide-bearing APCs and raise the immune response (Thery *et al.* 2002). Wolfer *et al.* using a human *in vitro* model further pointed out that exosomes secreted by living tumor cells, contained and transferred tumor antigens to DCs, which induced potent CD8+ T-cell-dependent anti-tumor effects on both syngeneic and allogeneic established mouse tumors. Skokos *et al.* also found that exosome sercreted by MCs induced immature DCs to up-regulate MHC class II, CD80, CD86, and CD40 molecules. Mature DCs acquired potent Ag-presenting capacity, which led to an elicitation of specific immune responses. So theoretically, DC-derived exosomes could substitute for DCs to elicit tumor rejection, and some preclinical data have highlighted their potential for the cancer immunotherapy.

Exosomes not only have function in up-regulation of immune system, but also in down-regulation. For example, in inflammatory conditions, secreted MHC class II carrying exosomes in blood are essential for the induction and maintenance of peripheral tolerance to non-self antigens from the intestinal content (Karlsson *et al.* 2001). The live sinusoidal endothelial cells or DCs, which had taken up these "tolerosomes", were conditioned by the hepatic microenvironment to produce IL-10 and express low levels of accessory molecules that would facilitate the development of regulatory T cells in response to the presented antigens aiming to avoid pathological inflammatory reactions.

Studies about argosomes, which are exosome-like vesicles, show that the transport of the morphogen Wingless through vesicles has very high membrane affinity suggesting that exosomes may play important role in developmental biology (Greco *et al.* 2001).

Indeed, exosomes may represent an entirely new mode of cell-cell communication. Uncovering their biogenesis mechanism and physiological role will extend a very exciting field in cell biology.

2. Research Aims

2.1 HYPOTHESIS:

After endocytosis, inwardly budding produces internal vesicles correlating with the autonomous protein and lipid synergetic sorting. To be specific, palmitoylation initiates tetraspanins oligomerization to form TEMs "pieces". TEMs help recruiting not only specific proteins (MHC, integrins, *etc*) through EC2 variable protein-protein interaction region, but also specific lipids (cholesterol, SM *etc*). Recruited lipids such as PI(3)P and $PI(3,5)P_2$ also help further recruit other proteins. Protein sorting and lipid sorting are synergetic. Pieces form polyhedral exosomes automatically in a topological manner, and cytoplasmic content is sealed passively. (Figure 1 and the legend give a detailed schematic description.)

2.2 SPECIFIC AIMs:

Aim.1 To exam the center role of tetraspanins in protein sorting:

1A. How do tetraspanins affect exosome formation?

1B. Are the interactions between EC2 domain and proteins important for sorting?

1C. Is palmitoylation required for forming highly ordered microdomains TEMs?

1D. How do functional TEMs drive lipid sorting after tetraspanins palmitoylation?

Aim.2 To exam synergetic lipid sorting based on TEMs:

2A. What is the role of PI(3)P and $PI(3,5)P_2$ in TEMs for protein sorting?

2B. Are lipids sorted into exosomes based on the properties of side chains?

2C. What is the function of cholesterol in the formation of the tetraspanin web?

2D. Does SM help the formation of rigid structure by decreasing membrane fluidity?

3. Experimental Proposal

3.1 Methods and Material Section

To prove the protein/lipid synergetic sorting model, both the morphology and the biochemical composition of exosomes will be studied in each experiment.

Morphologically, live-cell light microscopy (LCLM) will be used to study the dynamics, including exosome formation and component sorting. For example, the fluorescent phospholipid analog *N*-Rh-PE, which could be inserted into the plasma membrane and internalized (Willem *et al.* 1990), will be used as an exosome marker (Savina *et al.* 2003) *in vivo*. A confocal imaging system is required in LCLM, as the plasma membrane will generate a very intensive background with conventional epifluorescence microscopy.

The other morphological evaluation approach is immunoelectron microscopy (IEM). IEM data not only give a more detailed view, but also confirm the observation from LCLM. Ultrathin sections will be treated with antibody conjugated with colloidal gold and viewed with a transmission electron microscope (Raposo *et al.* 1996; Charrin *et al.* 2003). Micrographs will be prepared to a known scale to calculate the size of exosomes and MVBs.

To study exosomes at the biochemical level, purification of exosomes is very important. After collecting the supernatant from cell culture, exosomes will be isolated by differential centrifugation then flotation on 30% sucrose/D_2O, or by differential centrifugation then 0.2 μm filtration (Clayton *et al.* 2001; Andre *et al.* 2002; Clayton *et al.* 2004). After purification, both protein and lipid composition will be studied by various biochemical methods as described below.

To examine either integral membrane proteins or membrane-associated proteins, SDS-PAGE and fluorescence-activated cell sorting (FACS) will be employed (Thery *et al.* 2001). For FACS, defined amount of purified exosomes will be covalently linked to beads, and the presence of proteins will be revealed by immuno-fluorescence using FITC-conjugated antibody. Appropriate software will be chosen to gate only single beads for fluorescence analysis (Clayton *et al.* 2001).

In addition, lipids from both cells and exosomes will be extracted with chloroform/ methanol/water (1:1:1 v/v) in the presence of 1% ethanoic acid in methanol. The lower phase will be collected, and dried under nitrogen for mass spectrometry (MS) analysis. Thin layer chromatogram (TLC) with standard samples is applicable for lipids analysis as well (Laulagnier *et al.* 2004).

Exosomes are secreted by hematopoietic and epithelial cells. In order to test the protein/lipid synergetic sorting model, it is valuable to use different cell lines with various origins. K562 (ECACC no. 89121407) cells are human chronic myelogenous leukaemia cells deficient in MHC Class II, that can secrete exosomes (Johnstone 1996). Treatment of K562 cells with monensin, which is a Ca^{2+} ionophore, causes the formation of dilated MVBs and increased release of exosomes (Savina *et al.* 2003), which is very helpful in purification. This cell line can be stably transfected with exogenous constructs. The spleen-derived murine DC line D1 (Winzler *et al.* 1997; Thery *et al.* 1999); B-LDL, Epstein Barr virus immortalized B-lymphoblastoid cell line (Clayton *et al.* 2004) and human HEp-2 cells, which have epithelial morphology and secret exosomes (van Deurs *et al.* 1993; Rieu *et al.* 2000; Carloni *et al.* 2004), will also be used.

3.2 Protein sorting Section

Aim1A. How do tetraspanins affect exosome formation?

The tetraspanin family is comprised of at least 26 known human genes. CD9, CD63, CD81, CD82, CD151 are found in exosomes from various origins (Table 1). To study the dynamics and function of tetraspanins, it is valuable to make fluorescent constructs. It has been show that CD63-*N*-GFP was expressed properly in HeLa cells, and like CD63, distributed to late endocytic compartments (Lebrand *et al.* 2002). CD9, CD63, CD81, CD82, CD151 will be tagged with EGFP at the *N* terminus to transfect K562 cells, and LCLM will be used to obtain the data about the dynamics of tetraspanins during exosome formation. IEM will be used to confirm the data from LCLM by using anti-MHC II antibody to show the position of exosomes and anti-EGFP antibody to give the location of tetraspanins (Figure 3, Page 18). One important preliminary experiment is to use different antibodies to analyze the exact expression spectrum of tetraspanins on the purified exosomes from K562 cells.

[Alternative] Since EGFP is a bulky fluorescent protein, it may interfere with correct assembly and intracellular distribution of the chimeric molecules. It has been reported that recombinant proteins containing four cysteines can be fluorescently labeled in living cells by extracellular administration of 4',5'-bis(1,3, 2-dithioarsolan-2-yl)fluorescein (FLASH), which is membrane permeant and nonfluorescent until it binds with high affinity and specificity to the tetracysteine domain (Griffin *et al.* 1998). Tetracysteine will be added to the *N* terminus of tetraspanins, and FLASH will be added into the culture media.

According to the hypothesis, tetraspanins are the key regulators in exosome biogenesis. Different promoters will be used to increase the expression level of exogenous tetraspanins, and LCLM and IEM will be used to study the level of internal vesicles formation in MVBs and

exosomes secretion or possibly the enlargement of secreted exosomes. FACS data will confirm the morphology change of exosomes is due to the overexpression of tetraspanins (Figure 3).

Conversely, using immuno-trapping to inhibit the internalization of endogenous tetraspanins by incubating the cells with antibodies against tetraspanins (Figure 3). Antibody binding to proteins will interfere with their normal metabolism, which may leads to the decrease of exosome formation. There are many antibodies available against specific tetraspanins (Table 1). Four cell lines will be tested by immuno-trapping to give a broader idea.

[Alternative] RNAi is a technique that is used to specifically knock-down protein expression. Different siRNA sets will be designed according to the sequences of tetraspanins, and FACS data will confirm that the knock-down of tetraspanins causes the decrease of the exosome secretion. If siRNA is generated *in vivo* by expression hairpin RNA, K562 cells will be chosen. If siRNA needs to be microinjected into cells to have function, all four cell lines will be used.

Aim1B. Are the interactions between EC2 domain and proteins important for sorting?

The variable region of EC2 domain in tetraspanin is thought to function in protein-protein interaction (Hemler 2003; Stipp *et al.* 2003). In the protein/lipid synergetic sorting model, they are required for protein sorting in TEMs. Use gentle detergent such as CHAPS to lyse cell and immunoprecipitate (IP) with antibodies against tetraspanins. Integrins will be detected by immunoblotting. K562 cells will be transfected with constructs expressing the isolated EC2 fragment tagged with EGFP. LCLM will be used to locate the expression of EC2. Co-IP experiments will be performed to check whether less integrins are sorted to TEMs. FACS will detect the level of integrins associated with exosomes, which will be dramatically decreased when EC2 fragment is expressed (Figure 3, Page 18). IEM data will verify the diffused

integrins distribution during exosome formation, when competent fragments are expressed. It will be valuable to overexpress the EC1 or transmembrane domains as controls.

[Alternative] EC2 fragments will be purified from expressed bacteria, and microinjected into cells. LCLM/IEM/FACS will be used to study the change of integrins.

Aim1C. Is palmitoylation required for forming highly ordered microdomains TEMs?

The hydrogen bonds between tetraspanins transmembrane domains are very important for their stabilization, whereas the palmitoylation at specific intercellular sites are required for interactions between tetraspanins to form TEMs. It has been reported that simultaneous mutations of C11, C15, C242 and C243 eliminated >90% of CD151 palmitoylation, which was much more diffusely distributed and had markedly diminished stability during biosynthesis (Yang *et al.* 2002). And the complete palmitoylation-deficient mutant of CD151, which was mutated at C11, C15, C79, C80, C242 and C243, had markedly decreased association of with CD81 and CD63 (Berditchevski *et al.* 2002). The diffusion will be the result of the inefficient aggregation of TEMs.

Since there is no clear consensus site for palmitoylation of transmembrane molecules, which typically takes place at one or several cysteine residues located adjacent to or just within the transmembrane domain (Charrin *et al.* 2002), all Cys in candidate sites of tetraspanins will be mutated into Ser, and mutations will be tagged with EGFP to distinguish from endogenous proteins. K562 cells will be transfected with mutants. LCLM and IEM will be used to study the distribution of mutant tetraspanin in cells (Figure 3, Page 19). FACS with different antibodies against both tetraspanins and associated proteins will be chosen to indicate that mutations do not affect the overall conformation of tetraspanins. According to the hypothesis, highly ordered

TEMs are required for sorting lipids and disruption of palmitoylation influences the formation of TEMs, the lipid composition of purified exosomes will be analyzed.

[Alternative] Since palmitoylation increases the hydrophobicity of protein (Linder and Deschenes 2003), mutate Cys into Trp at the candidate sites might be done to mimic the palmitoylation effect. K562 cells transfected with these mutants may release more exosomes, as TEMs will be formed without the modifying step.

Aim1D. How do functional TEMs drive lipid sorting after tetraspanins palmitoylation?

Using the same experimental strategy in Aim1C, focus on the lipid sorting here. Inhibition of palmitoylation will influence the formation of TEMs, which sequentially affect lipid sorting (Figure 3, Page 19).

3.3 Lipid sorting Section

Aim2A. What is the role of PI(3)P and PI(3,5)P_2 in TEMs for protein sorting?

Lipids recruited in TEMs also help protein sorting in the model, which includes PI(3)P and PI(3,5)P2. It has been reported that PI(3)P helps recruit cytoplasmic effector proteins that have either a FYVE domain or a PX domain and might participate in vesicle formation (Stenmark and Gillooly 2001; Birkeland and Stenmark 2004). All four cell lines will be treated with wortmannin, which inhibits PI3K activity, and LCLM and IEM will be used to see whether internal vesicles formation in MVBs and exosome secretion are affected. SDS-PAGE and silver staining will be employed to compare drug treated and untreated samples to see whether some components are sorted inappropriately upon inhibition of PI3K activity. Analysis lipids in purified exosomes will verify that the miss sorting of specific proteins results from the absence of PI(3)P (Figure 3, Page 20).

[Alternative] Function-blocking antibodies against PI(3)P 5 Kinase might be microinjected to study the influence on protein sorting as well.

Aim2B. Are lipids sorted into exosomes based on the properties of side chains?

It has been shown that specific lipids are sorted into exosomes (Laulagnier *et al.* 2004). Endocytic organelles sorted membrane lipid components efficiently based on their preference for association with domains of varying characteristics (Mukherjee *et al.* 1999). Fluorescent lipids with different side chains will be ordered from Molecular Probes Inc., and cells will be labeled with an appropriate dilution of fluorescent lipids for 2 min at 37^{o}C. According to the protein/lipid synergetic sorting model, only lipids with ordered structure and high hydrophobicity will be sorted into MVBs and exosomes. LCLM will be used to analyze the sorting of fluorescent lipids to exosomes (Figure 3, Page 20).

Aim2C. What is the function of cholesterol in the formation of the tetraspanin web?

It has been shown that cholesterol is involved in the formation of microdomains in the cell (Mukherjee and Maxfield 2000; Mobius *et al.* 2002). Tetraspanin complexes were resistant upon depletion of cholesterol, whereas the number tetraspanin-containing vesicles decreased (Claas *et al.* 2001). These results give the assumption that cholesterol is required in the interaction between TEMs and functions in the formation of the tetraspanin web.

To study cholesterol, biotinylated and non-cytolytic perfringolysin O (BCΘ) will be used to label cholesterol in both living cells and cryosections of fixed cells (Mobius *et al.* 2002). Anti-biotin antibodies and gold-protein A will be used for detection.

All four cell lines will be treated with methyl-β-cyclodextrin (MβCD), a drug which does not incorporate into the membrane but extracts membrane cholesterol by including it in a non-polar cavity (Charrin *et al.* 2003). MβCD treatment may interrupt the formation of exosomes

by interfering with the tetraspanin web formation. Purified exosomes will be analyzed by IEM and FACS both morphologically and biochemically (Figure 3, Page 21).

[Alternative] The tetraspanin-associated cholesterol might not be easily reached by MβCD. Cells will be treated with hydrophobic amines such as U18666A (Biomol Research Lab., PA), which can cause cholesterol accumulation by blocking cellular cholesterol transport. Increased cholesterol may facilitate the tetraspanin web formation, which may generate either lager amount or bigger size of exosomes. FACS and IEM will be used to analyze the morphology changes, and TLC data with cholesterol standard will verify the increase of cholesterol.

Aim2D. Does SM help the formation of rigid structure by decreasing membrane fluidity?

SM is found to be highly enriched in exosomes (Laulagnier *et al.* 2004). It has a high frequency of saturated amide-linked acyl chains and has a fairly cylindrical molecular shape, which is a rigid structural component (Goni and Alonso 2002; Ramstedt and Slotte 2002).

Since sphingomyelinase (SMase) catalyzes the hydrolysis of sphingomyelin into ceramide and phosphorylcholine, which are more fluid and permeable (Goni and Alonso 2002), specific SMase inhibitors, such as scyphostatin, alutenusin, xanthone, *etc*, will be added into the cell culture, and the size of MVBs and secreted exosomes will be measured by IEM. If SM functions in exosome formation, less SMase, more SM will generate larger rigid regions, which means larger exosomes. The lipid components of exosomes will be analyzed to check the results from the increase of SM.

[Alternative] If the inhibitors don't work well in the four cell lines, the analogues of SM, modified by methylene (Hakogi *et al.* 2000) or difluoromethylene (Yokomatsu *et al.* 2001), will be used. Cells incubated with these SM analogues, which not only mimic SM function but also inhibit SMase activity, may generate larger exosomes.

It has also been pointed out that cholesterol interacts favorably with SM to form a liquid-ordered phase, which has a role in the formation of microdomains (Slotte 1997). Experiments in Aim2C will also generate smaller exosomes if cholesterol and SM function together.

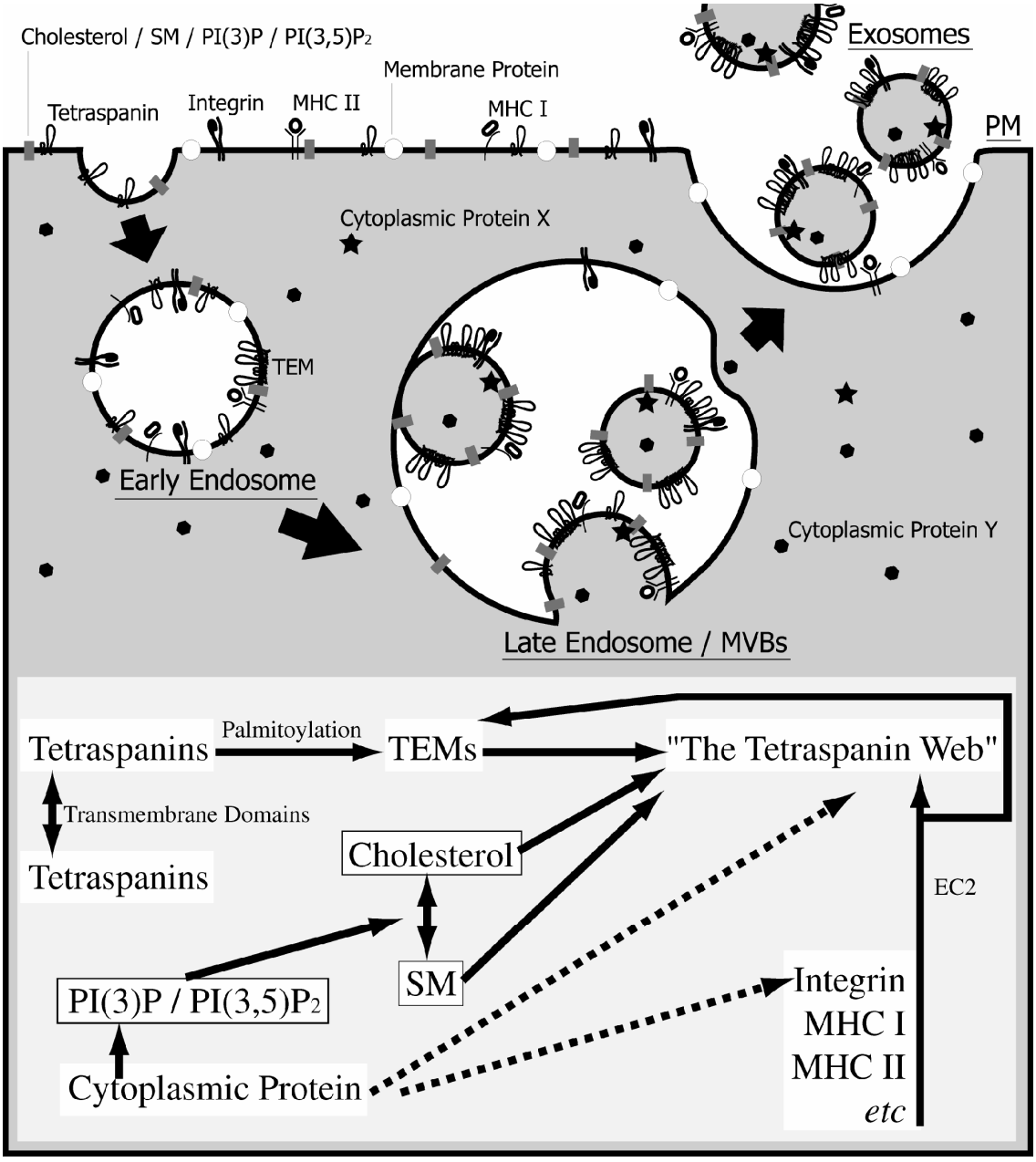
4. Alternative Model

The polypeptide ubiquitin acts as a regulated sorting signal at different steps of biosynthetic pathways. One simple model described the ubiquitin-dependent sorting machinery is that endocytic cargo carrying a ubiquitin signal is recognized by novel ubiquitin binding proteins and transported into specific sub-cellular organisms. Studies about the sorting mechanism of carboxypeptidase S (Katzmann *et al.* 2001; Katzmann *et al.* 2004) and Vps10 (Urbanowski and Piper 2001) showed that ubiquitin is a general signal used as a ticket for entry into vesicles budding into the MVB lumen. It has been pointed out that EGFR has tyrosine kinase activity, can phosphorylate and activate c-Cbl, which is an E3 ubiquitin ligase and required in mono-ubiquitination protein sorting. Some MVBs were found to be enriched with EGFR (Katzmann *et al.* 2002), which hints the possibility that protein sorting within MVBs is mechanistically conserved and monoubiquitination sorting protein targeted into lysosome might work as monoubiquitination sorting protein targeted into secreted exosomes too. If serial experiments in Aim1 do not work as predictions and give contrary results, it is reasonable to consider the possibility that monoubiquitination plays a more central role in the protein sorting during exosome biogenesis.

About the lipid sorting, “like dissolves like rule” plays the central role. Highly ordered microdomain favorites lipids with long and saturated side chains, and lipid-ordered phase helps stabilize proteins.

Figures and Tables

Figure 1. Exosome biogenesis model

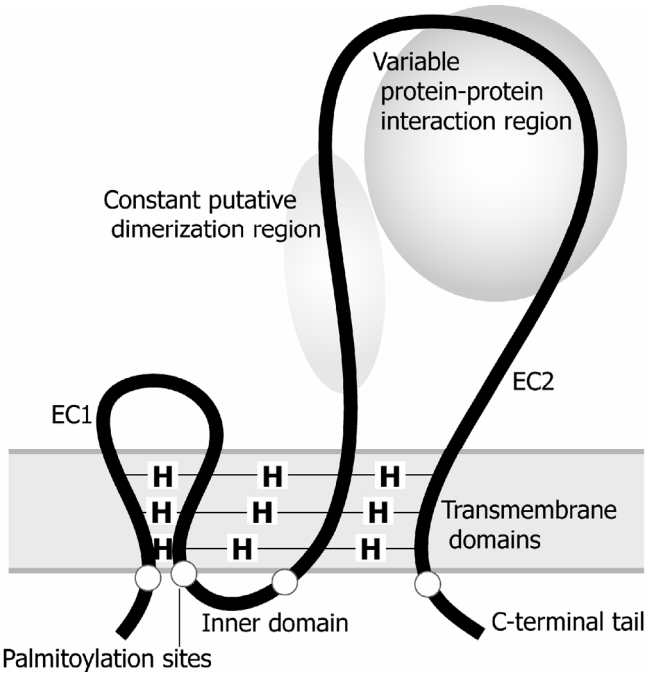


After endocytosis, tetraspanins on the membrane of early endosomes are palmitoylated and aggregrate into TEMs. TEMs recruit specific membrane proteins (MHC I, MHC II, integrins *etc*) through EC2 variable protein-protein interaction regions. Highly ordered structure in TEMs helps recruit specific lipids as well (grey rectangle in the upper panel; cholesterol, SM *etc*). Extend protein-protein interactions and protein-lipid interactions form “the tetraspanin

web" and stabilize TEMs, which function as pieces to assemble vesicles in a topological manner. Lipids in TEMs recruit other lipids such as PI(3)P and PI(3,5)P_2 following "like dissolves rule". PI(3)P and PI(3,5)P_2 further sort specific cytoplasmic protein (black star in the upper panel) into inwardly budding vesicles in MVBs. During the autonomous formation of vesicles, some cytoplasmic proteins (black hexagon in the upper panel) are sealed passively. After MVBs fuse with the plasma membrane ("PM" in the upper panel for short), exosomes are released into the extracellular milieu. Membrane proteins not sorted onto the exosomes (white circle in the upper panel) are returned to the plasma membrane.

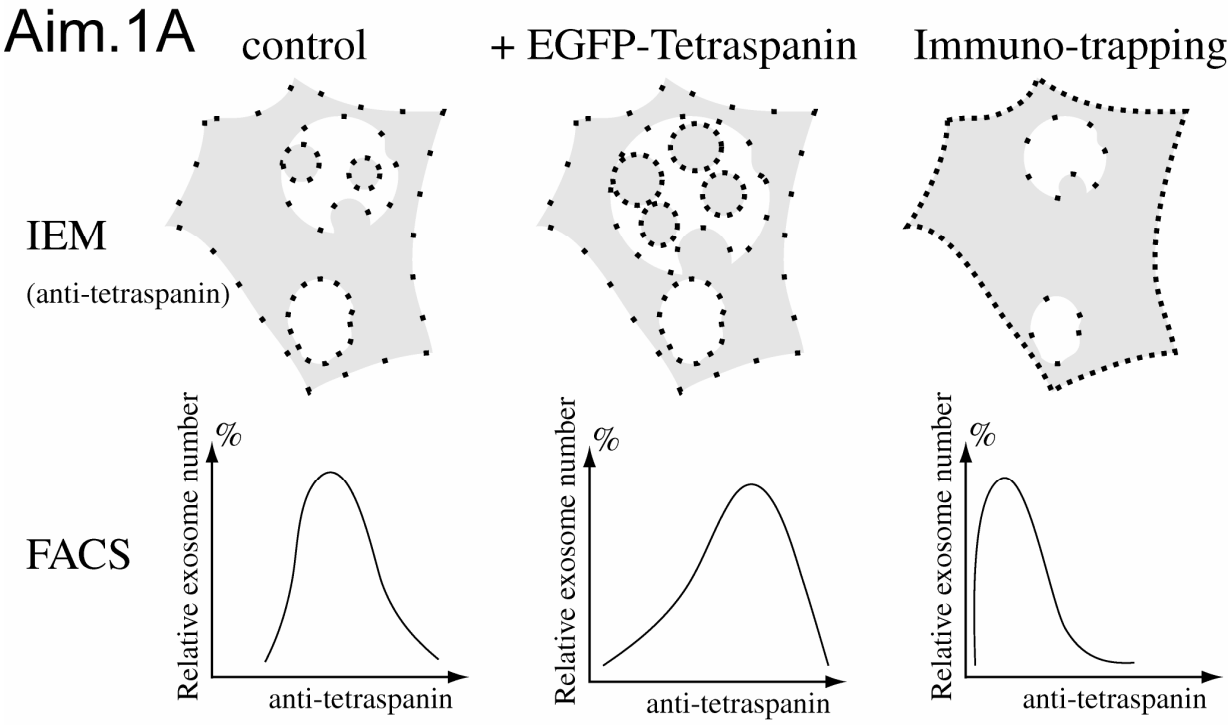
The lower panel gives a schematic diagram showing that the protein sorting and lipid sorting during exosome biogenesis are synergetic.

Figure 2. Functional domains in tetraspanin

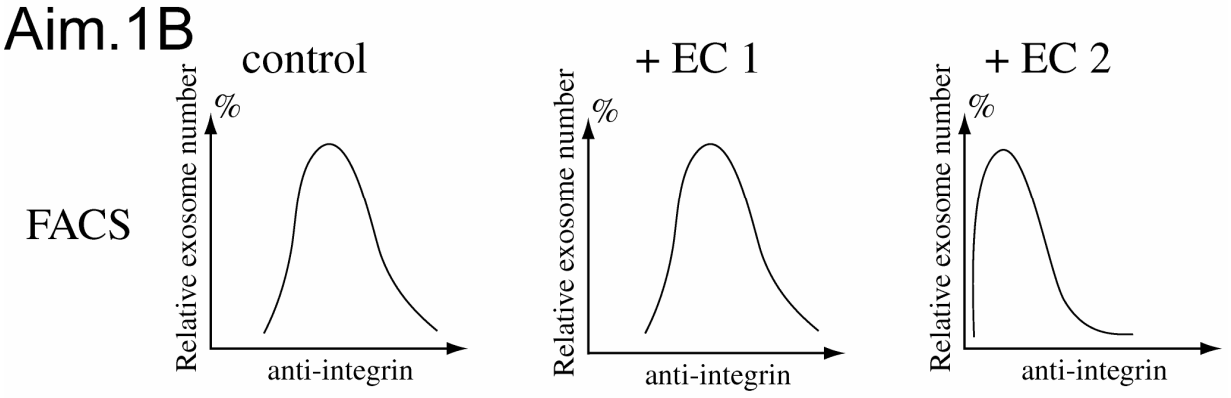


The large extracellular loop (EC2) is subdivided into variable and constant regions. The small extracellular loop (EC1), transmembrane domains (regions linked by "H"), inner domain, palmitoylation sites (white circle) and C-terminal tail are also present in the figure.

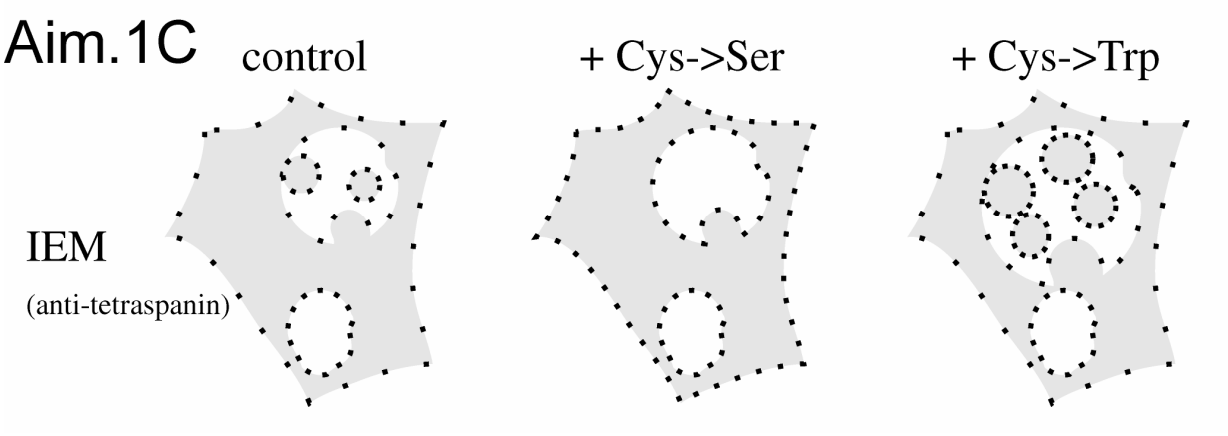
Figure 3. Expected results



How do tetraspanins affect exosome formation?



Are the interactions between EC2 domain and proteins important for sorting?



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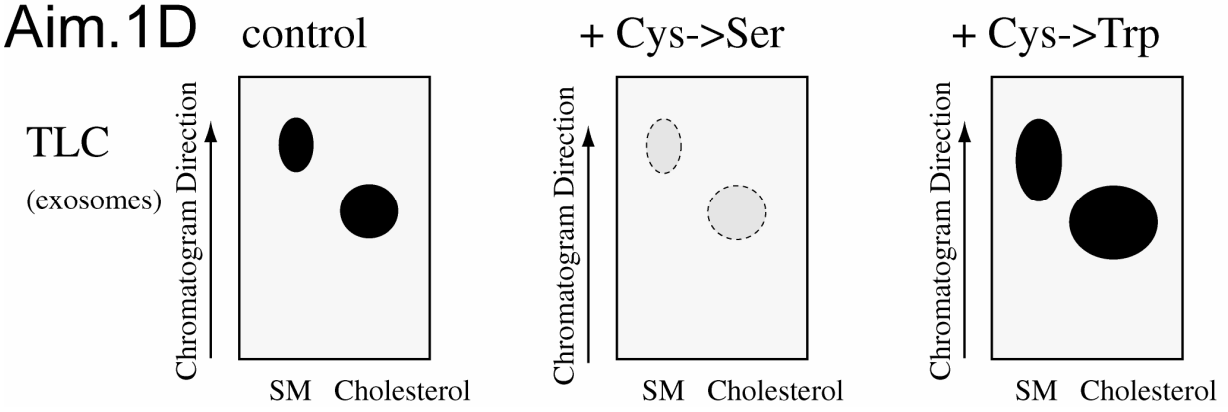

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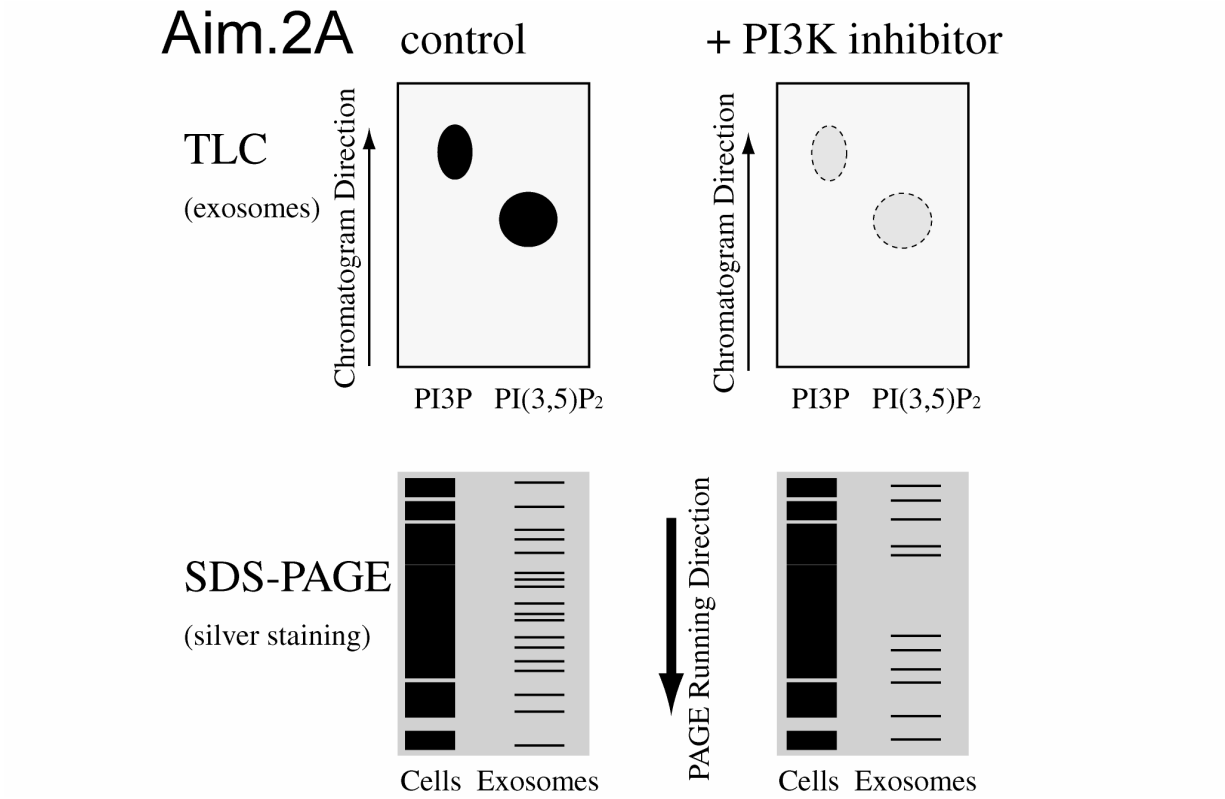
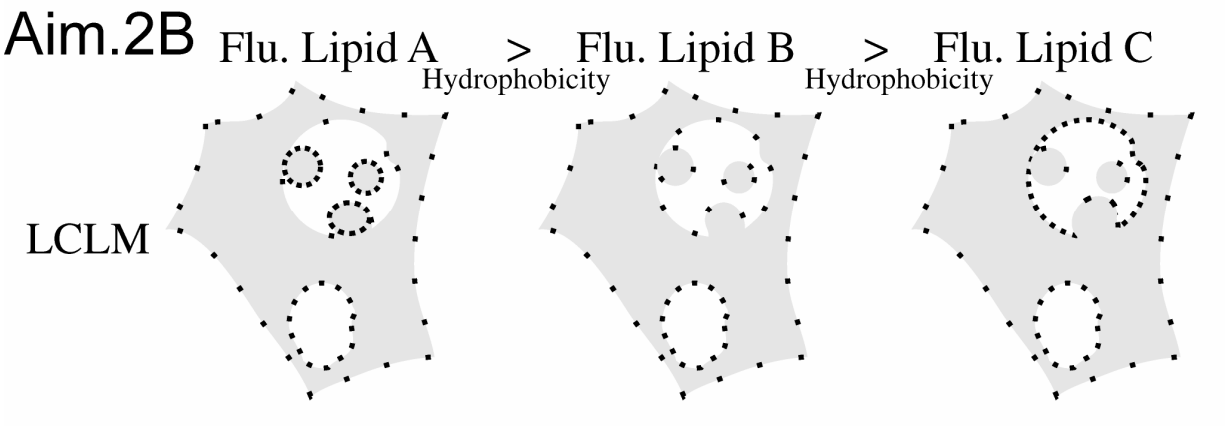
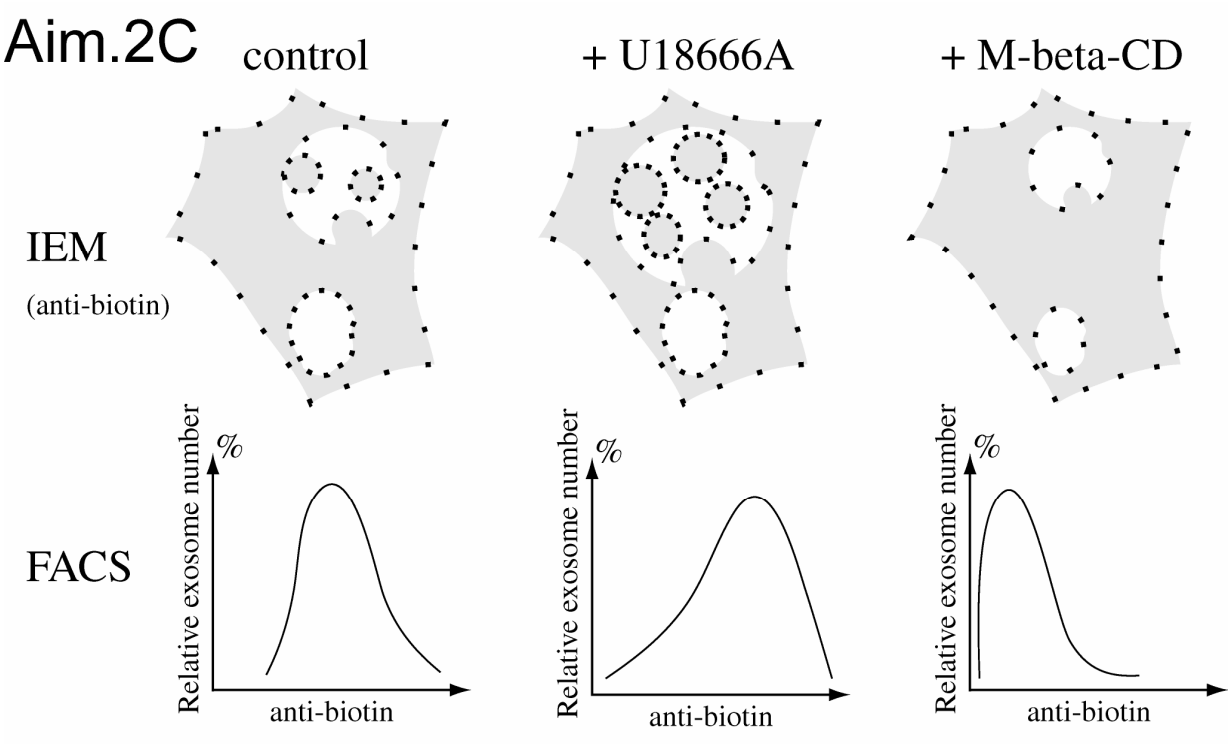


What is the role of PI(3)P and PI(3,5)P_2 in TEMs for protein sorting?



Are lipids sorted into exosomes based on the properties of side chains?



What is the function of cholesterol in the formation of the tetraspanin web?

Table 1. Tetraspanins present in exosomes from various cell lines

Tetraspanin	Monoclonal Antibodies [1]	Cell Line			
		K562 [2]	D1 [3]	B-LDL [4]	HEp-2 [5]
CD9	C9-BB, DU-ALL-1, SYB-1, ALB-6, BA-2, 50H19, mab7	?	+	+	?
CD63	2C6, 1B5, H5C6, 6H1, CLB-gran/12, RUU-Sp2.28	+	+	+	?
CD81	M38, Z81, TS81, 1D6, 5A6, JS64, JS81, 4TM-1	+	?	+	+
CD82	TS82, 1A4, 4F9, C33	+	?	+	?
CD151	TS151, 11B1G4, 5C11	?	?	+	?

[1] Taken from references and http://www.ncbi.nlm.nih.gov/prow/.

[2] Taken from (Mannion *et al.* 1996; Savina *et al.* 2002; Savina *et al.* 2003).

[3] Taken from (Zitvogel *et al.* 1998; Thery *et al.* 1999).

[4] Taken from (Escola *et al.* 1998).

[5] Taken from (Carloni *et al.* 2004).

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