# The ins and outs of mutlivesicular bodies

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# Chapter 6

## Summary and perspectives

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*Review in preparation*

## Mapping the MVB commute

The multivesicular organization of late endosomal compartments is no novelty, but the multitude of purposes it serves is trending more than ever. Indeed, the importance of the multivesicular body in the context of intercellular communication has recently sparked a renewed interest in its functional attributes and the luminal vesicles it contains. In past studies, attention has been focused primarily on intraluminal vesicle formation, especially the mechanisms governing ESCRT-mediated invagination and fission. It is now abundantly clear that MVBs function as sorting platforms for cargoes of diverse fates. Nevertheless, which aspects, both intrinsic and environmental, dictate the ultimate destination of their ILVs —and therefore sorted cargoes— remains poorly defined. A greater knowledge of such aspects would significantly advance our understanding of exosome turnover and allow for manipulation of exosome-mediated processes. Along these lines, the findings described in my thesis draw a clearer picture of the different pathways emanating from MVBs, aka the routes ILVs may travel through, and the molecular mechanisms involved. The relevance of such avenues in immunity was explored in Chapter 1, where I reviewed the intricate rearrangements of the endolysosomal system in the course of MHC class II – mediated antigen presentation and summarized the fates of ILVs in the MHC-II compartment of immature and mature antigen-presenting cells. Retrofusion stood out as a potentially crucial process for the transit of MHC-II molecules to the cell surface upon immune cell maturation. Despite its prior implication in viral infection and extracellular vesicle transfer, retrofusion of ILVs has until recently not been directly studied. To address this knowledge gap, in Chapters 2 and 3, I described the design and use of an innovative assay to circumvent the technical challenges posed by monitoring such a dynamic intraorganellar process. While we were able to successfully measure (and perturb) ILV retrofusion within MVBs, subsequent attempts at detection of exosome retrofusion in acceptor cells, summarized in Chapter 4, were unsuccessful. In Chapter 5, our focus shifted to the beginning of exosomes' journey, particularly to the control various enzymes involved in endosomal processes exercise over exosome release. Lastly, in this Summary and Perspectives Chapter I will discuss in more depth the relevance of the findings evoked above for the vesicular biology landscape and consider the future prospects arising from these and other studies on the exosome scene.

## Retrofusion, or how to escape multivesicular bodies

In Chapter 2 and 3[1], I described the first chemically tunable and temporally controlled system to demonstrate the occurrence of retrofusion in MVBs. Exploitation of the system allowed us to identify cellular features relevant to this dynamic membrane process and ultimately relate it to exosome formation. We found that augmentation of either endolysosomal pH or lipid concentration in MVB membranes resulted in a significant impediment of retrofusion. Furthermore, ectopic expression of Interferon-induced-transmembrane (IFITM) proteins – specifically IFITM3 – significantly hampered retrofusion of ILVs (Figure 1), which could be related to their antiviral function[2-4]. Interestingly, viral fusion is also known to require low pH[5] and can be interfered with through manipulation of endocytic lipid composition[6-8]. These

findings indicate the existence of mechanistic parallels between retrofusion and viral infection. Moreover, our retrofusion-monitoring assays led us to conclude that not all ILVs present in mature endosomes can fuse back to the limiting membrane, implying coexistence of retrofusion-competent ILVs with retrofusion-inert ILVs, the latter of which are likely destined for either degradation or exocytosis. In support of this assertion, hampering retrofusion rendered the exosome pool more inert and revealed a delicate equilibrium between exocytosis and retrofusion. The molecular characteristics branding ILVs for either of these pathways are still unknown, e.g. whether exosomes eventually acquire the ability to fuse with acceptor cells remains to be determined.

The aforementioned findings have brought us to the conclusion that distinct pools of ILVs can be present not only in the same cell but even within the same MVB. While we now know that at least three types of ILVs exist—those carrying cargoes for lysosomal degradation[9], those en route to becoming exosomes[10], and now also retrofusing ILVs[1], a detailed understanding of the attributes that dictate these different fates still lays ahead. A major question to address in this context is the role of ILV composition with respect to endolysosomal membrane residents (lipids and proteins alike) as well as cargoes transiting through the MVB. One clue to solving the molecular puzzle of retrofusion may lie in the regulation of endosomal luminal pH. Specifically, fusion of certain viral particles with endosomal membranes requires acidic pH within host endocytic compartments, as well as the presence of fusogenic proteins and often proteases [5, 11], and our findings on constitutive ILV retrofusion also demonstrate responsiveness to pH. From the exosome perspective, it is valuable to note that ILVs targeted for exocytosis appear mostly inert to retrofusion prior to their release from donor cells and yet are capable of retrofusion upon uptake by recipient cells[12]. Whether exosomes possess (part of) the machinery required to achieve retrofusion already at the ILV stage, and how such machinery could be regulated to license future membrane fusion events remains to be examined. Interestingly, endosomal pH correlates with endosome positioning[13] – the closer late compartments reside relative to the perinuclear area, the more acidic their luminal pH becomes. Based on this, it can be expected that MVBs en route to the plasma membrane would feature pH prohibitive of retrofusion, thereby preserving ILVs for exocytosis. On the other hand, maturation of MVBs towards proteolytic lysosomes, typically sequestered near the nucleus, would then be supportive of retrofusion to ensure ILV cargo recycling before impending degradation. Given these considerations, it may be important to consider spatiotemporal organization of endolysosomal constituents as we strive to understand what attributes support exosome uptake and fusion in recipient cells seeing to obtain information from donors.



**Message in an exosome: how to detect delivery**

Intercellular communication underlies coordinate responses of cells to one another and is thus instrumental for proper tissue function. Exosomes, which can be derived from various membrane sources, represent a key pipeline used to convey information in the form of genetic and proteinaceous materials between cells[10] and the cellular goods they transmit have been implicated in a variety of physiological

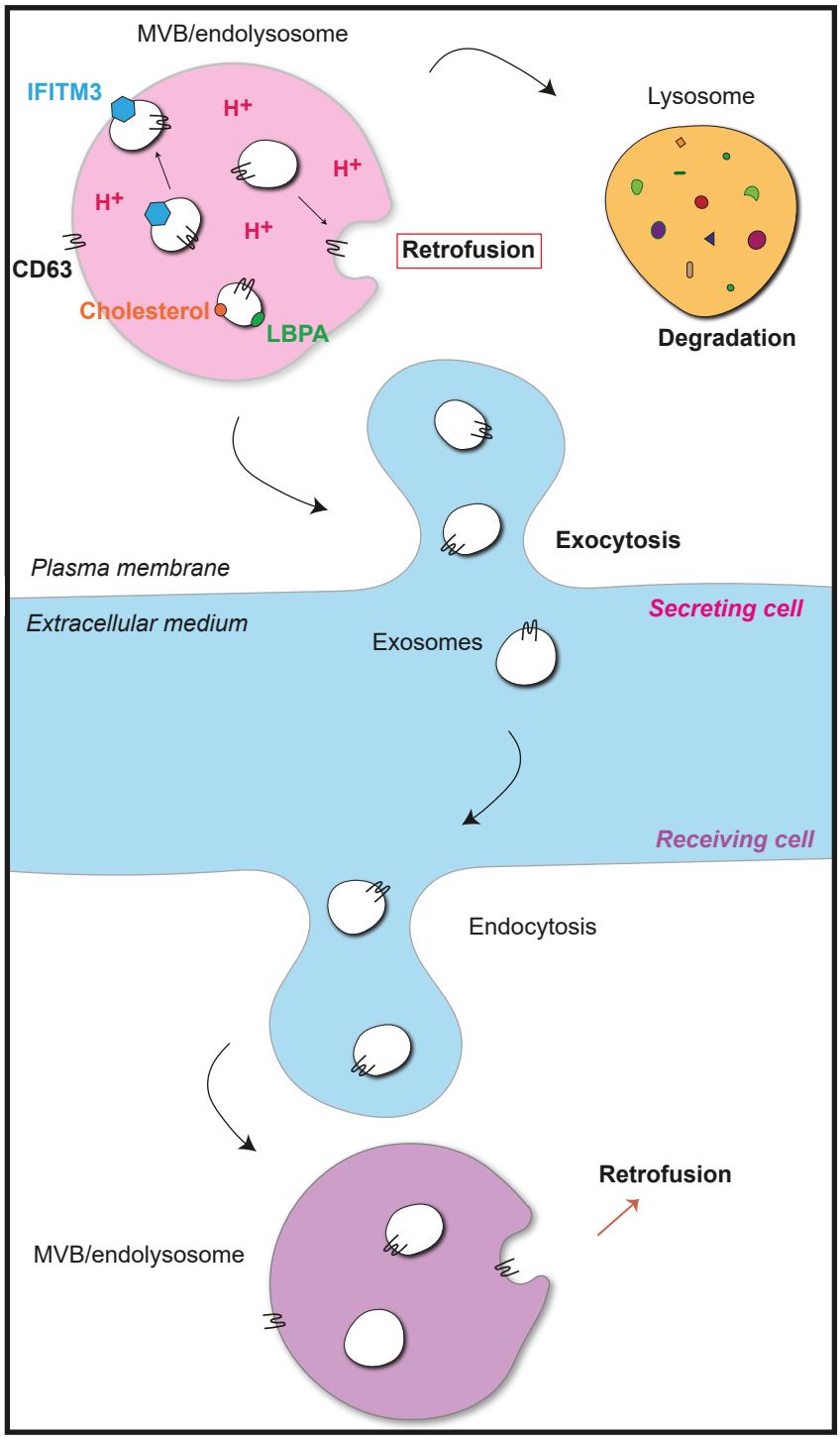
MVB/endolysosome
IFITM3
H+
H+
H+
H+
CD63
Cholesterol
LBPA
Retrofusion
Lysosome
Degradation
Exocytosis
Plasma membrane
Extracellular medium
Secreting cell
Exosomes
Receiving cell
Endocytosis
Retrofusion
MVB/endolysosome

**Figure 1. Exosome turnover: from secretion to uptake.**
*Schematic representation of the intercellular journey travelled by exosomes and the diverse fates ILVs encounter from within the MVB: degradation upon MVB fusion with a lysosome, retrofusion, or secretion in the form of exosomes. Retrofusion depends on intraluminal pH as well as membrane lipid composition (cholesterol, LBPA) and can be hampered by interferon-induced proteins such as IFITM3. Upon secretion, exosomes may travel through extracellular medium and be endocytosed by recipient cells. Progression to late compartments can then result in retrofusion of exosomes and release of their content into the cytosol of recipient cells.*

processes[14]. With a wide range of transferrable cargoes, exosomes can serve as instruments of progression in pervasive human pathologies[15], such as cancer [16] and neurodegeneration [17]. Against this backdrop, the need to understand the cell biological attributes of exosome release and uptake is more pressing than ever. Following their release from donor cells, exosomes may travel through extracellular space and be taken up by acceptor cells. When endocytosed, exosomes can transit to late compartments and eventually transfer their content to the cytosol of receiving cells[10] and have a biological effect on these. Since neither lysosomal degradation nor de novo secretion would lead to this outcome, it has been suggested that exosomes perform retrofusion in order to escape the MVB and discharge their cargo (Figure 1). A recent study by Bhagyashree S. Joshi et al. in fact ruled out any other pathway that may be responsible for exosome uptake[12]. Therefore, understanding the process of exosome retrofusion within MVBs is crucial to deciphering the inner workings of exosome transfer between cells and developing ways to modulate the molecular mechanisms involved.

Retrofusion is a unique process that takes place in the opposite direction (relative to the intra/extracellular divide) as compared to other fusion processes occurring within the cell. What we know about endosomal fusion mostly comes down to the action of cytosolic SNARE proteins[18], which are absent from the lumen of MVBs. Intra-endosomal fusion must therefore be operated by a different set of molecules that have yet to be discovered. Intent on identifying such machinery, independent attempts have been made to design elaborate systems for direct detection of exosome fusion within recipient cells[19, 20]. On the heels of our microscopy-based retrofusion-monitoring system, we attempted to develop a bioluminescence-based assay relying once again on the enrichment of CD63 on ILVs to study exosome uptake but were unable to detect desired fusion events (Chapter 4). In a similar fashion, a recent study showcased a reporter-gene assay[21] where exosomes were equipped with tetracycline transactivator (tTA)-fused tetraspanins. Upon exosome uptake, the tTA was to be cleaved from CD63 - or CD81, CD9 - by the TEV protease, inducing luciferase reporter gene expression in recipient cells. However, transfer of exosome contents could only be observed in the presence of the Vesicular Stomatitis Virus glycoprotein (VSV-G), which likely enhances fusion. Similarly, in cellular models used to observe propagation of prions or pathogenic Tau aggregates involved in neurodegenerative disorders, expression of VSV-G in donor cells led to enhanced aggregate induction by extracellular vesicles in recipient cells[22]. The reasons why assays lacking a VSV-G boost do not perform well in the context of exosome uptake remain unclear, though the inefficiency of exosome uptake might

be the most logical explanation. Evidence supporting this hypothesis was recently provided in a study by Bonsergent et al. in which exosome cargo Hsp70 was fused to the NanoLuc luciferase. Exosome uptake was assessed by measuring luciferase activity in the cytosolic fraction of acceptor cells following cell fractionation[23]. While this was quite an indirect technique to measure exosome uptake, the findings obtained implied exosome fusion is dependent on endolysosomal pH and can be hampered by the presence of IFITM proteins, corroborating the results we showed in Chapter 3. Interestingly this study suggested the limiting factor in exosome uptake is not exosome retrofusion but internalization by recipient cells. Inefficient endocytosis would indeed render detection of exosome fusion more challenging – the amount of engulfed exosomes and therefore fusion events would be insufficient. This would explain why most methods developed to monitor exosome fusion within acceptor cells have failed. New and innovative approaches will likely be needed to fully expose the intricacies of exosome transfer between cells, enabling its exploitation for targeted therapeutic delivery and opening avenues for opposing this process in disease.

**Expanding the exosome pool**

Identifying perturbations allowing manipulation of exosome secretion is a critical endeavor to further our understanding exosome-mediated intercellular communication. In Chapter 5 we show that three enzymes involved in endolysosomal dynamics and positioning are instrumental to exosome secretion. We revealed RNF26 depletion enhances exocytosis while loss of USP15 hampers exosome release. The tight control of RNF26 and USP15 over endolysosomal architecture is likely connected to their influence over exocytosis, though other pathways relying on the respective activities of RNF26 and USP15 might be involved as well. Interestingly, the enzyme USP32, in charge of Rab7 deubiquitination, also effects exosome production. The ubiquitination state of Rab7 must be carefully handled in order to ensure late compartments functions, such as fission of recycling tubules or progression toward lysosomes. Consequently, impairment of such processes through loss of USP32 potentially resulting in a block in lysosomal degradation, likely presses the cell to evacuate its endosomal contents through exocytosis. Finally we investigated the effect of the above perturbances on exosomal microRNA (miRNA) secretion, as miRNAs are a typical and physiologically relevant exosome cargo. We found that loss of RNF26, which boosts exocytosis, strongly enhances miRNA release in exosomes. It is still unclear if only exosome production is increased under these conditions or if miRNA sorting into ILVs is also amplified. To conclude, we found new ways of boosting exosome secretion through enzyme-dependent pathways and conjecture such modifications may be used in further studies of exosome transfer between cells as well as in utilization of exosomes for therapeutic delivery.

Altogether in this thesis I explored the ins and outs of the multivesicular body, the pathways that intersect at this central endocytic compartment, the dynamics governing its lumen and how such processes impact various fields of cell biology and disease.

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