

Retrofusion: Escape route out of the endosome

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Summary

Intraluminal vesicles (ILVs) accumulate within the endosomal lumen before lysosomal delivery or extracellular release. Perrin et al. [1] have developed an elegant assay showing that escape from the endosome lumen is possible by retrofusion of ILVs with the endosomal limiting membrane.

Intraluminal vesicles (ILVs) are small vesicles that bud from the limiting membrane of endosomes into the lumen of multivesicular endosomes/bodies (MVBs). Many are subsequently delivered to the lysosome, providing a means to degrade endocytosed membrane proteins. The paper by Perrin et al. [1] demonstrates that sorting of membrane proteins onto ILVs is not irreversible and ILVs can retrofuse with the limiting membrane. They use an elegant assay involving timed dimerization of a split cytoplasmically expressed protease that, when induced to dimerise, cleaves an inserted site in the cytoplasmic domain of the ILV marker, CD63 (Figure 1). That more endosomal CD63 becomes accessible to cleavage than is present on the limiting membrane of the MVB provides direct evidence for retrofusion and an assay that allows the requirements for retrofusion to be tested.

A number of lines of evidence have previously suggested that retrofusion can occur. Mannose-6-phosphate receptors (M6PR) cycle between the Golgi and endosomes to deliver newly synthesised acid hydrolases to the endocytic pathway but M6PR is not delivered to lysosomes. Surprisingly immuno-gold EM revealed that M6PR could be found on ILVs [2], suggesting that escape from the ILV, presumably by retrofusion, might be possible. In dendritic cells (DCs) MHCII molecules were found concentrated on ILVs in unstimulated cells, but after stimulation peptide-loaded MHCII trafficks to the cell surface [3], again suggesting the possibility of ILV retrofusion. However more recent data indicates that MHCII on ILVs in unstimulated DCs is destined for lysosomal degradation and is a different population from the newly synthesised MHCII that presents antigen on the cell surface after activation [4]. While EGF-stimulated EGFR is considered a marker of the canonical ILV pathway to the lysosome, stress can also induce EGFR endocytosis and P38 MAP kinase-dependent retention of the receptor in perinuclear endosomes, where EGFR can be found on ILVs [5]. Addition of P38 inhibitor after stress-induced EGFR endocytosis resulted in EGFR return to the cell surface, consistent with retrofusion. A number of studies have also suggested that retrofusion can occur in pathological situations. For example, the Anthrax toxin lethal factor is sorted onto ILVs before delivery to its site of action in the cytosol [6]. Vesicular stomatitis virus favours fusion with the membrane of ILVs over the endosomal limiting membrane, implying

retrofusion before delivery of the nucleocapsid into the cytosol [7]. The small size of ILVs (some <40nm diameter) means that they can only be visualised by electron microscopy, making their budding and fusion impossible to observe in living cells. Thus, the findings of Perrin et al. [1] are important because they show that retrofusion can occur, whilst further suggesting that resistance to retrofusion can control ILV fate.

Lysosomal degradation is not the only potential destination for ILVs. In pigmented cells PMEL-derived filaments polymerise on ILVs, eventually forming the striations upon which melanin is deposited to form melanosomes [8]. ILVs can also be released into the extracellular space upon fusion of MVBs with the plasma membrane. Now termed 'exosomes' they have been implicated in many processes, including intercellular communication, antigen presentation, prion transmission and tumor metastasis. Perrin et al. [1] show that a proportion of ILVs are resistant to retrofusion and this population are selectively enriched in the ILVs that are released as exosomes, suggesting that retrofusion may allow proteins and lipids to escape from the exosome pathway. This intriguing observation raises many questions.

Are lysosomally directed ILVs resistant to retrofusion too and, if so, how are they segregated from those released as exosomes? Sorting of ubiquitinated cargo like EGF-stimulated EGFR onto ILVs is thought to remove them from the recycling pathway. Thus, it would make sense for the lysosomally directed ILV population to be retrofusion resistant. Adapting the protocol of Perrin et al. to measure retrofusion of EGFR-containing ILVs could allow this to be tested but the question of how lysosomally and exosomally directed cargo or ILVs are segregated remains. ILV sorting of lysosomally directed cargo depends on the ESCRT machinery, a series of protein complexes that recognise ubiquitinated cargo like EGF-stimulated EGFR, concentrating them into domains on the limiting membrane of the MVB and then promoting ILV formation [9]. ILVs destined for release as exosomes may be formed by both ESCRT-dependent and independent mechanisms [10]. Lysosomally directed and exosomally directed cargoes may be delivered to distinct MVBs. For example, in DCs ubiquitinated MHCII undergo ESCRT-dependent ILV sorting in MVBs that fuse with lysosomes, whilst non-ubiquitinated MHCII undergo non ESCRT-dependent ILV sorting in MVBs for release as exosomes [11]. EGF-stimulated EGFR en route to the lysosome are trafficked in a subset of MVBs [12], whilst stress-induced EGFR undergo WASH-dependent segregation into a distinct population of MVBs [5]. ILVs formed by different mechanisms can also co-exist in the same MVB [13] or on contiguous membranes [14], indicating that segregation mechanisms for ILVs with different destinations must exist.

What is the mechanism of retrofusion? Importantly, ILV retrofusion requires the fusion of luminal faces of the membrane and so is topologically distinct from intracellular fusion events that are mediated by cytoplasmically facing SNAREs. Retrofusion was reduced by elevation of lysosomal pH, cholesterol accumulation or accumulation of the unconventional phospholipid lysobisphosphatidic acid (LBPA) [1]. These treatments all influence LBPA, which has been strongly implicated both in retrofusion and fusion of endocytosed viruses [15, 16]. Elevating lysosomal pH, which also inhibits viral fusion, has been shown to disrupt the fusogenic properties of LBPA [15], while LBPA and cholesterol transport are intimately related. Inhibition of the lysosomal cholesterol export protein, Niemann Pick type C-1 (NPC1) results in accumulation of LBPA as well as cholesterol [15]. Moreover, LBPA was shown to interact with NPC2, increasing efficiency of sterol transfer to the limiting membrane [15, 16].

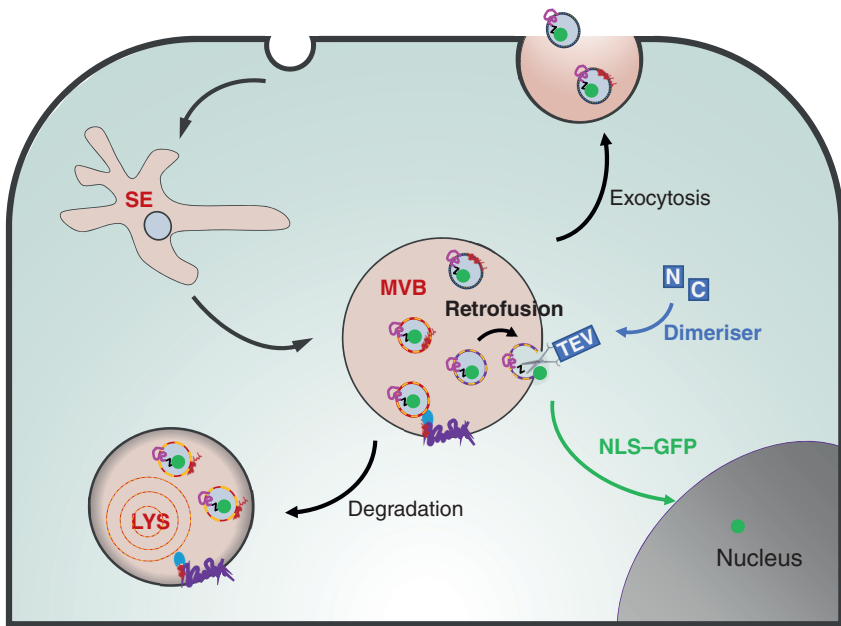
Although LBPA has previously been shown to promote retrofusion, Perrin et al. [1] found that treatment with Thioperamide maleate, which results in LBPA accumulation [15], impaired retrofusion. Similarly, while LBPA promotes viral fusion with late endosome membrane, elevated LBPA reduced infectivity of HIV [17], though the underlying mechanism remains unclear. LBPA is enriched on ILVs and lysosome internal membranes, with very little found on the limiting membrane [18], yet promotes retrofusion of ILVs/viruses through interaction with a cytosolic ESCRT-associated protein, ALIX [15, 16]. Thus, trapping LBPA in the endosome lumen by treatment with thioperamide maleate or by NPC1 inhibition, may result in impaired recruitment of cytosolic ALIX with a downstream effect on retrofusion. LBPA can also promote ILV formation through interaction with ALIX [15, 16]. If retrofusion increases LBPA accessibility to ALIX, might it be rapidly followed by inward vesiculation to deliver any lysosomal targeted proteins, that might have inadvertently returned to the limiting membrane, back onto ILVs in a continuous and dynamic sorting process? Interestingly, LBPA has recently been implicated in ALIX-mediated delivery of tetraspanins onto ILVs destined for exocytosis [19], although several studies have found no enrichment of LBPA on exosomes [20], consistent with high concentrations of LBPA at the lysosome where it promotes sphingolipid catabolism. Though many unknowns remain, the lipid environment clearly impacts retrofusion and endosomal sorting and importantly, the assay reported by Perrin et al. provides a way to begin to probe the molecular mechanisms regulating retrofusion.

Why are some ILVs resistant to retrofusion? ILVs often appear to be on strings, suggesting that they are not freely diffusible within the MVB lumen and so might have limited access to the limiting membrane. In melanogenic cells the amyloidogenic processing that generates fibrils upon ILVs [8] might be sufficient to prevent retrofusion. Could resistance to retrofusion be regulated by the limiting membrane in addition to the ILVs themselves? The MVB limiting membrane has ESCRT coated regions, tubulating regions, actin coated regions and regions where it forms membrane contact sites with other organelles. Could these features regulate availability for retrofusion? In the lysosome the glycocalyx formed from the high concentration of LAMPs on the limiting membrane may inhibit retrofusion, thus ensuring that those ILVs that are delivered to the lysosome following MVB:lysosome fusion remain in the degradative lumen.

What is the function of ILV retrofusion in healthy cells? Retrofusion of ILVs carrying signaling receptors could allow those receptors to interact with cytoplasmic substrates and allow, for example, stress-induced EGFR to participate in survival signalling [5]. Could retrofusion allow retrieval of proteins destined for recycling to the Golgi or plasma membrane that have been included on ILVs of lysosomally directed MVBs as bystanders? Establishing ways to specifically inhibit ILV retrofusion without interfering with upstream endocytic trafficking will allow the elucidation of the full functions of ILV retrofusion.

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	Retrofusion-competent ILVs		TCS-CD63		Lysosome membrane + glycocalyx
	Exocytosed ILVs		NLS-GFP		NPC1
	Degradation-targeted ILVs		FRB-N-TEV		Cholesterol
	LBPA-rich membrane		FKBP-C-TEV		