ELSEVIER

Protein sorting into multivesicular endosomes

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Multivesicular endosomes are important as compartments for receptor downregulation and as intermediates in the formation of secretory lysosomes. Work during the past year has shed light on the molecular mechanisms of protein sorting into multivesicular endosomes and yielded information about the machinery involved in multivesicular endosome formation. Monoubiquitination functions as a signal for sorting transmembrane proteins into intraluminal vesicles of multivesicular endosomes and subsequent delivery to lysosomes. A molecular machinery that contains the ubiquitin-binding protein Hrs/Vps27 appears to be central in this sorting process. Three conserved multisubunit complexes, ESCRT-I, -II and -III, are essential for both sorting and multivesicular endosomes formation. Enveloped RNA viruses such as HIV can redirect these complexes from multivesicular endosomes to the plasma membrane to facilitate viral budding.

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Abbreviations

EGF	epidermal growth factor
ESCRT	endosomal sorting complex required for transport
FYVE	conserved in Fab1p/YOTB/Vac1p/EEA1
LBPA	lyso-*bis*phosphatidic acid
MVE	multivesicular endosome
LRP	low-density lipoprotein receptor related protein
PI3P	PtdIns 3-phosphate
PtdIns	phosphatidylinositol
STAM	signal-transducing adaptor molecule
TGN	*trans*-Golgi network
UIM	ubiquitin-interacting motif
Vps	vacuolar protein sorting

Introduction

The 'multivesicular body' was described by electron microscopists some 50 years ago ([1•] and references therein) as an organelle that consists of a limiting membrane enclosing many (sometimes several hundred) internal vesicles of 40–90 nm. Later work has shown that multivesicular bodies represent endocytic intermediates (Figure 1), and here we will refer to them as 'multivesicular endosomes' (MVEs). MVEs are formed from sorting (early) endosomes and thus contain molecules that have been internalised through endocytosis. They also receive biosynthetic cargo from the *trans*-Golgi network (TGN), including precursors of lysosomal enzymes. In most cell types, early MVEs (also called endosomal carrier vesicles) mature into — or fuse with — late MVEs (also called late endosomes), which ultimately fuse with lysosomes. The sorting of transmembrane proteins into topologically distinct limiting and intraluminal membranes (Figure 2) has been proposed to serve several important functions. First, transmembrane proteins in the intraluminal membrane will be susceptible to degradation by lysosomal hydrolases, whereas proteins in the limiting membrane are resistant because they only expose their luminal region (which is usually protease-resistant, owing to extensive glycosylation). Second, intraluminal vesicles might represent storage vehicles for transmembrane proteins that are to be released from the cell in a regulated manner. Third, receptor signalling is, at least in principle, possible from the limiting membrane of MVEs, but not from the membranes of intraluminal vesicles. This means that sorting into MVEs can determine both the delivery of transmembrane proteins to lysosomes and the extracellular space, and also the ability of endocytosed receptors to transmit signals.

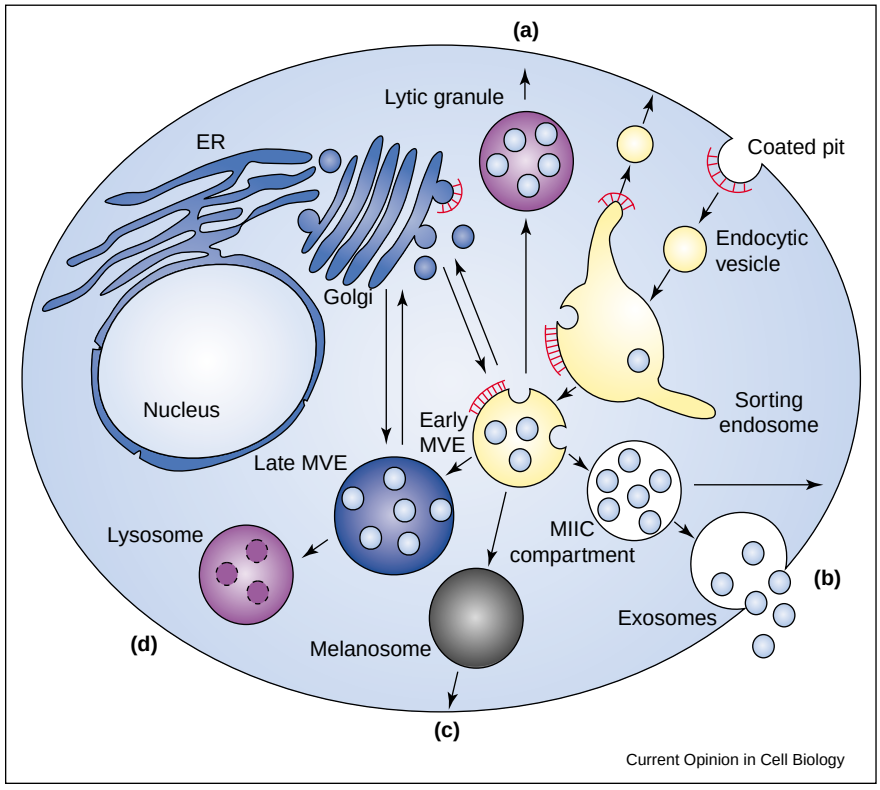
In specialised cell types, for example melanocytes and haematopoietic cells, MVEs serve as intermediates in the formation of secretory lysosomes, such as melanosomes, MHC II compartments and lytic granules [2•] (Figure 1). The formation of these specialised organelles requires sorting of specific proteins at the level of MVEs. Upon an appropriate stimulus, secretory lysosomes fuse with the plasma membrane, and any intraluminal vesicles will be shed into the extracellular space or transferred to neighbouring cells. In the case of antigen-presenting cells, such vesicles are referred to as exosomes, which have attracted profound interest as vehicles of immunomodulation [3•,4•]. Exosome-like vesicles can also be used as carriers for morphogens, as demonstrated by the finding that MVE-derived vesicles known as argosomes mediate dispersion of the morphogen Wingless over large distances in *Drosophila* imaginal discs [5].

In this review, we will highlight some recent studies, published in 2002 and 2003, that shed light on the molecular mechanisms of the sorting into and formation of MVEs.

Ubiquitin as a sorting signal for multivesicular endosomes

To understand the molecular mechanisms of receptor downregulation, a key issue has been to define the signals

Figure 1



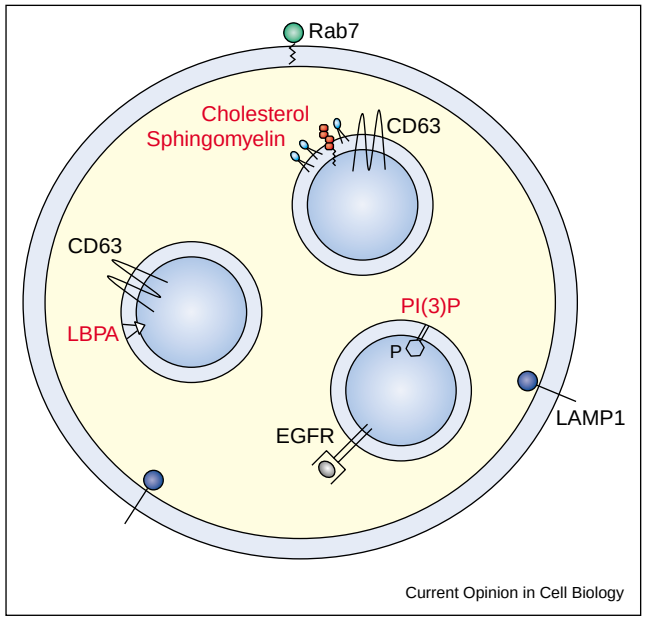
Formation and functions of MVEs. Multivesicular endosomes are formed after invagination of the limiting membrane of the sorting endosome. They are versatile and can serve different functions in different cell types, such as being precursors for **(a)** lytic granules in T lymphocytes, **(b)** MHC class II compartments and exosomes in antigen-presenting cells, **(c)** melanosomes in melanocytes, and **(d)** late MVEs/lysosomes in most nucleated cells. Both endocytic and biosynthetic proteins are sorted in and out of MVEs, indicated by arrows. Clathrin-coated buds are found at the plasma membrane, the TGN and on tubular regions of the sorting endosome, whereas flat clathrin coats are found on early endosomal compartments. Clathrin is indicated in red.

that target transmembrane proteins into intraluminal vesicles of MVEs. Several studies have shown that mono-ubiquitination can serve as a signal for both endocytosis and MVE sorting [6–10] (Table 1), and recombinant fusion of Golgi-, plasma-membrane- or endosome-located transmembrane proteins with ubiquitin has been shown to act as a strong signal for lysosomal targeting [11,12,13•].

We are now beginning to learn how monoubiquitin-mediated protein sorting works. An important clue came from a bioinformatical screen for proteins containing a motif that mediates binding of the proteasomal subunit S5a to ubiquitin. Such a ubiquitin-interacting motif (UIM) was identified in several known regulators of endocytosis, including epsin and Eps15, as well as several putative regulators of MVE sorting [14]. This finding was quickly followed up by experimental studies that showed that UIMs do bind ubiquitin [13•,15••,16, 17•–20•]. Moreover, the UIMs of Ent1/Ent2 (yeast homologues of epsin) are important for endocytosis of the α-factor receptor, which is known to be ubiquitin-dependent [19•]. Likewise, the UIM of the early-endosomal protein Hrs is required for sorting of a transferrin-receptor–ubiquitin fusion protein to the degradative pathway [13•], and the UIMs of the yeast Hrs homologue Vps27 are required for sorting of endocytosed α-factor receptor, newly synthesised carboxypeptidase S and a ubiquitin fusion reporter construct to the vacuole [18•,19•]. These findings suggest that UIM-containing proteins might interact with ubiquitinated cargo at the plasma or early endosome membrane and thereby mediate its sorting into endocytic or intraluminal vesicles.

Even though this is an attractive hypothesis, several complications have to be considered. First, UIMs only bind ubiquitin with low affinity (K_d values in the high micromolar range), and it is unlikely that this interaction alone is strong enough to mediate efficient sorting [13•,20•]. It is striking, however, that both epsin and Hrs are complexed to other UIM-containing proteins (Eps15 and Eps15–STAM [signal-transducing adaptor molecule], respectively) [18•,19•,21], so perhaps multi-UIM-containing

Figure 2



Schematic representation of an MVE. Multiple proteins and lipids have been detected on internal vesicles and on the limiting membrane of multivesicular endosomes. It is not clear, however, if all these molecules play a physiological role in MVEs. The molecules indicated here represent a selection of proteins and lipids that are considered typical candidates found in late MVEs [3•,42•].

sorting complexes bind with high avidity to multiubiquitin-containing receptor complexes. Second, all UIM-containing endocytic proteins tested (Epsin, Eps15, Eps15R, Hrs and STAM) become monoubiquitinated upon receptor activation [20•,22], and in principle the UIM could be a signal for monoubiquitination rather than a sorting determinant. More likely, the monoubiquitination serves to regulate the sorting functions of UIM-containing proteins, for example by sterically controlling interactions with cargo or sorting components or by facilitating formation of large complexes through multiple UIM–ubiquitin interactions. Third, even if UIM-containing proteins do function in receptor sorting, they may not necessarily interact directly with cargo — interactions might be indirect via ubiquitinated adaptor proteins. Fourth, UIM-containing proteins are not the only ubiquitin-binding proteins with a putative role in endocytic sorting. Yeast Vps23 and its mammalian homologue Tsg101 contain a UEV (ubiqutin E2 variant) domain, which, like the UIM, binds ubiquitin with low affinity [23•]. Since Vps23 and Vps27 (Hrs) function in vacuolar sorting of the same ubiquitinated cargo [24], this raises the question as to how their interactions with cargo could be coordinated. Genetic evidence suggests that Vps27 functions upstream of Vps23 [1•], raising the possibility that Vps27 might capture ubiquitinated cargo initially and subsequently deliver it to Vps23 (see below). However, the functional relationship between these two ubiquitin-binding proteins needs to be clarified.

Table 1

Examples of transmembrane proteins sorted into MVEs via ubiquitin (Ub).

Protein	Ub facilitates endocytosis	Ub facilitates MVE sorting	Refs
EGF receptor	?	Yes	[36]
Interleukin-2 receptor	No	Yes	[7]
CXCR4	No	Yes	[8]
β2 adrenergic receptor	Yes*	Yes	[9]
Growth hormone receptor	Yes*	Yes	[37]
LRP	No	Yes*	[39•]
ENaC	Yes	Yes	[10]
Ste2 (Sc)	Yes*	Yes	[6]
Ste3 (Sc)	Yes†	Yes†	[62•]
CPS (Sc)	‡	Yes	[24]
Phm5 (Sc)	‡	Yes	[12]
Hmx1 (Sc)	‡	Yes	[12]

CPS, carboxypeptidase S; CXCR4, a chemokine receptor; ENaC, epithelial sodium channel; Hmx1, haem oxidase; Phm5, a polyphosphate phosphatase; Sc, *S. cerevisiae*; Ste2, α-factor receptor; Ste3, a-factor receptor. *Sorting requires ubiquitination of accessory components. †True for constitutive receptor trafficking. Ligand-induced endocytosis and recycling is ubiquitin-independent. ‡Directly sorted to MVEs from TGN.

Hrs and ubiquitinated proteins are found in a flat clathrin coat on endosomes

Epsin and Hrs, two UIM-containing proteins that function, respectively, at the plasma membrane and at the early endosome, share several features. They both contain a phosphoinositide-binding domain; they both bind Eps15; and they both bind clathrin. The clathrin-binding ability of epsin is easy to reconcile with its role in clathrin-dependent endocytosis [25]; however, the significance of the clathrin-binding ability of Hrs is more elusive. Hrs is localised to endosomal microdomains that contain a flat, 'bilayered' clathrin-containing coat morphologically distinct from the budding clathrin coat found on endosomal tubules [13•,26•]. Overexpression of Hrs recruits clathrin to endosomes, whereas wortmannin (a phosphatidylinositol 3-kinase inhibitor) treatment, which causes Hrs to dissociate from endosome membranes, leads to a dissipation of the bilayered coat [26•,27]. This suggests that Hrs might function as a clathrin adaptor on endosomes. However, as the bilayered clathrin coat does not appear to form buds, what is its function? The finding that ubiquitinated membrane proteins are enriched in this coat suggests that it could play a role in endosomal retention of ubiquitinated membrane proteins before their inclusion into intraluminal vesicles of MVEs [13•,26•]. Since clathrin has not been detected inside intraluminal vesicles by electron microscopy, the bilayered coat either has to dissociate before endosomal invagination, or invagination occurs at sites adjacent to the coat.

Ubiquitin ligases that mediate multivesicular endosome sorting

The covalent attachment of ubiquitin to protein lysine groups is a complex process that ultimately requires the activity of substrate-specific ubiquitin ligases [6]. Numerous ubiquitin ligases, which are characterised by RING or HECT domains, are expressed in yeast and mammalian cells. Although we know little about the protein determinants that dictate the specificity of most ubiquitin ligases, there are many examples of their importance in endocytosis, MVE sorting and receptor signalling. In yeast, the HECT-domain-containing ubiquitin ligase Rsp5 is important for endocytosis of the G-protein-coupled α-factor receptor Ste2 (probably by ubiquitinating a *trans*-acting factor) [28] and for MVE sorting of the amino acid permease Gap1 [29]. A transmembrane RING-domain-containing ubiquitin ligase, Tul1, ubiquitinates Golgi proteins that have polar transmembrane domains, and signals their entry into MVEs [30•]. In *Drosophila* and vertebrates, the Notch–Delta receptor pair, which among other things controls neuronal differentiation, is regulated via endocytosis and MVE sorting. At least four ubiquitin ligases are known to ubiquitinate these proteins and to regulate their activity [31•]. *Drosophila*/*Xenopus* Neuralised and zebrafish Mind Bomb ubiquitinate Delta, thus causing its endocytosis and degradation [32–34,35•], whereas mouse Itch/AIP4 and Sel-10 (and their respective *Drosophila* and *Caenorhabditis elegans* homologues) regulate Notch in a similar fashion [31•].

Signalling by epidermal growth factor (EGF) receptors is likewise attenuated by ligand-induced endocytosis and MVE sorting, as demonstrated by the finding that EGF receptor signalling in *Drosophila* is increased when MVE formation is impaired [15••]. The RING-domain-containing ubiquitin ligase Cbl appears to play a major role in MVE sorting of the activated EGF receptor by binding to the autophosphorylated receptor and causing its ubiquitination [36].

The existence of multiple ubiquitin ligases with distinct substrate specificities illustrates that spatiotemporal control is essential for protein ubiquitination and sorting.

Crosstalk between proteasomal and lysosomal protein degradation

Polyubiquitinated proteins are targeted to proteasomes for proteolysis, whereas monoubiquitinated proteins are targeted to lysosomes or vacuoles [6]. However, a surprising connection between proteasomal and lysosomal degradation has been identified recently. Proteasome inhibitors prevent lysosomal targeting of growth hormone and EGF receptors and the multifunctional receptor LRP (low-density lipoprotein receptor related protein), and they do so by excluding the receptors from entering intraluminal vesicles of MVEs, without affecting MVE formation as such [37,38,39•,40•]. In principle, proteasome inhibitors might inhibit MVE sorting by reducing the available pool of ubiquitin, but this does not appear to be the case, as EGF receptors are efficiently ubiquitinated even in the presence of proteasome inhibitors [40•]. Moreover, while proteasome inhibitors inhibit endocytosis of growth hormone receptors, they are without effect on endocytosis of EGF receptors and LRP [39•]. It is possible that unknown proteins might serve as negative regulators of MVE sorting, and that these are inactivated by polyubiquitination and proteasomal degradation. There is indeed evidence that polyubiquitinated proteins are associated with endosomes [17•]. An antibody specific for conjugated polyubiquitin stains Hrs- and clathrin-containing microdomains on early endosomes [21], suggesting that polyubiquitinated proteins might be localised to the bilayered endosomal clathrin coat.

It is worth noting that polyubiquitin might also function as an MVE targeting signal. The yeast general amino acid permease Gap1 becomes polyubiquitinated in the TGN under nutrient-rich growth conditions, and this signals its entry into MVEs [6]. Since the monoubiquitinated receptor is sorted from the TGN to the plasma membrane, this suggests the existence of sorting machineries in the TGN that can distinguish between mono- and polyubiquitinated membrane proteins.

Non-ubiquitinated membrane proteins can also be sorted to multivesicular endosomes

Although monoubiquitin is an important sorting determinant for MVEs, non-ubiquitinated transmembrane proteins also can be sorted into intraluminal vesicles. This is true for yeast Sna3 (whose function is not known) [12], as well as for mammalian LRP. The latter protein nevertheless appears to require the ubiquitin system, since its MVE sorting is inhibited by proteasome inhibitors [39•]. On the other hand, Sna3 is efficiently targeted to MVEs even when the levels of unconjugated ubiquitin are reduced [12]. These results suggest that although ubiquitin is a versatile signal for MVE targeting, alternative targeting mechanisms also exist.

The MVE sorting machinery that recognises non-ubiquitinated proteins is not known, but GASP (G-protein-coupled receptor-associated sorting protein), represents a possible component because it binds to non-ubiquitinated δ-opioid receptors and targets them into MVEs [41•].

Lipids and multivesicular endosome sorting

The fact that intraluminal membranes have different lipid compositions than limiting membranes [3•,42•] (Figure 2) indicates that lipids are sorted along different routes to the membranes of MVEs. This suggests that protein sorting into MVEs might, at least in part, depend on lipids. Consistent with this, phosphatidylinositol (PtdIns) 3-phosphate (PI3P), which is formed through phosphorylation of PtdIns by a class III PtdIns 3-kinase, is

important for several steps of endocytic trafficking, including MVE formation [43,44]. This lipid is found on limiting and intraluminal endosomal membranes [45], and it recruits proteins containing phox homology or FYVE (conserved in Fab1p/YOTB/Vac1p/EEA1) domains to endosomes [43,46]. Hrs is an example of a FYVE-domain protein that is recruited to endosomes via PI3P binding [47]. Because the absence of Hrs inhibits formation of MVEs [15••], similar to effects seen when PI3P formation is inhibited [44], a major role of PI3P in MVE formation might be to recruit Hrs. This does not rule out the possibility, however, that other PI3P binding proteins, such as the PI3P 5-kinase Fab1/PIKfyve, might also regulate MVE sorting [1•].

While PI3P functions by recruiting cytosolic proteins to endosomal membranes, other lipids could have a more direct effect by creating local membrane environments that favour MVE sorting and formation of intraluminal vesicles. It is interesting to note that tetraspanins and glycosylphosphatidylinositol-linked proteins, which are known to associate with cholesterol- and sphingolipid-rich 'rafts' [48], are enriched in intraluminal vesicles, and exosomes have been found to contain high levels of cholesterol and sphingomyelin [3•]. Clustering of proteins into lipid rafts could thus be linked to their intraluminal sorting. While sphingomyelin and cholesterol are candidate lipids for mediating protein sorting into MVEs, an unusual phospholipid, lyso-*bis*phosphatidic acid (LBPA), is a candidate for mediating inwards invagination of endosomal membranes. Because of its unusual structure, LBPA is inefficiently degraded by lipases, and it accumulates in intraluminal vesicles of late MVEs [49]. Antibodies against LBPA (which are observed in patients suffering from Antiphospholipid syndrome) inhibit MVE formation, suggesting that LBPA does play a role in this process. The inverted-cone shape of LBPA might favour the inward invagination required to form MVEs. It is worth noting, however, that LBPA has not yet been identified in yeast. This lipid might thus participate in an alternative mechanism of MVE formation found only in higher cells.

ESCRT complexes are required for sorting and multivesicular endosome formation

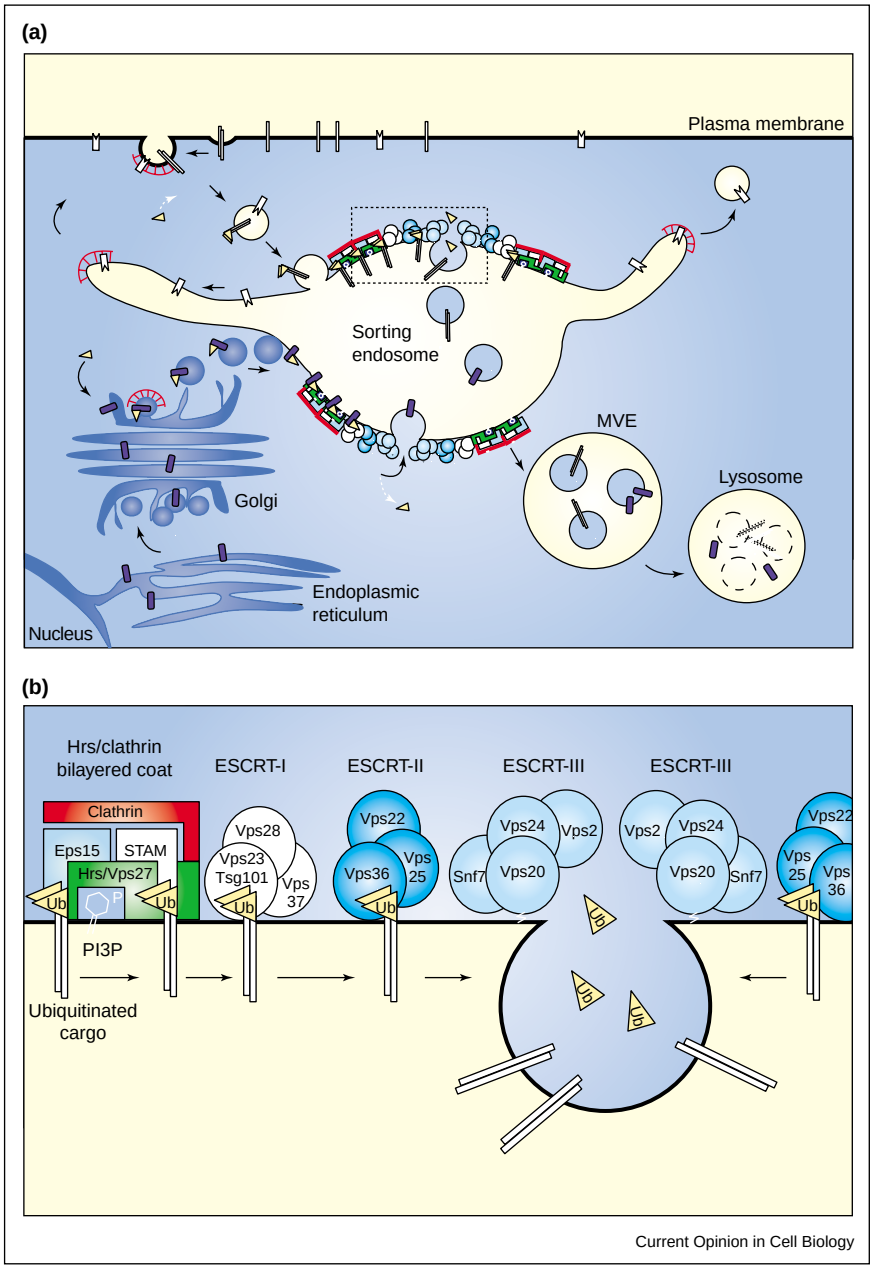
Electron microscopic evidence suggests that the intraluminal vesicles of MVEs are formed through invagination and pinching-off of the endosome membrane [1•]. Since this vesicle-budding process occurs in the opposite orientation compared with other budding events of cellular membranes (i.e. outwards from the cytosol), it is evident that the molecular mechanisms must be different from the well-characterised mechanisms of, for instance, coated-vesicle budding at the plasma membrane. A screen for vacuolar protein sorting (*vps*) mutants in yeast has proven highly successful in identifying the basic machinery for MVE formation [1•]. The so-called class E subgroup of *vps* mutants, which currently comprise 17 members, is characterised by the absence of intraluminal vesicles in the vacuole (such vesicles become evident in wild-type yeast when vacuolar hydrolase activity is blocked). The class E mutants are further distinguished by a cup-shaped multilamellar organelle, the class E compartment [24]. This compartment is thought to represent an endosome that is unable to form intraluminal vesicles.

Recent work has demonstrated that the Vps class E proteins can be found in several subcomplexes, including Vps27–Hse1 [18•] and the 'endosomal complexes required for transport', ESCRT-I, ESCRT-II and ESCRT-III [24,50••,51•] (Figure 3). ESCRT-I consists of three subunits (Vps23, Vps28 and Vps37) and binds ubiquitinated proteins via the Vps23 subunit (see above). This complex is therefore likely to be involved in the sorting of ubiquitinated proteins, possibly downstream of Vps27–Hse1. ESCRT-II, which also contains three subunits (Vps22, Vps25 and Vps36), appears to function downstream of ESCRT-I, since its overexpression can suppress the loss of ESCRT-I (but not *vice versa*) [1•]. Intriguingly, even this complex contains a ubiquitin-binding subunit, Vps36, which binds ubiquitin via its NZF (Np14 zinc finger) domain [52].

The ESCRT-II complex is recruited transiently to endosome membranes and is required for the membrane recruitment of ESCRT-III, suggesting that ESCRT-II functions upstream of ESCRT-III. The latter complex consists of two subcomplexes (Snf7–Vps20 and Vps2–Vps24) which seem to be recruited consecutively to membranes. The membrane dissociation of the ESCRT complexes is controlled by the AAA-type (chaperone-like) ATPase Vps4. In addition, a few other Vps class E proteins (Fti1, Vps60, Bro1 and Vps44) are involved in MVE formation, although their relationship with the ESCRT complexes is not known [1•]. With the exception of Vps37, mammalian equivalents of all Vps class E proteins have been identified, indicating that these complexes serve a conserved function. Indeed, Tsg101 and hVps28, the human homologues of Vps23 and Vps28, are found on endosomes and are required for lysosomal trafficking of endocytosed EGF [17•].

The fact that both Vps27–Hse1 (Hrs–STAM), ESCRT-I and ESCRT-II bind ubiquitin suggests that ubiquitinated cargo can be relayed between these complexes as part of the sorting process (Figure 3). But how do the ESCRT complexes mediate inward vesiculation of endosomal membranes? Their subunit structures do not leave many clues. Perhaps the most striking feature is that all the ESCRT-III subunits are small coiled-coil proteins. This is reminiscent of another group of small coiled-coil proteins, the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, which mediate membrane docking and fusion through the formation

Figure 3



Model for protein sorting into MVEs. **(a)** Schematic overview of the sorting of ubiquitinated membrane proteins from endocytic and biosynthetic pathways into multivesicular endosomes and lysosomes. Ubiquitin (Ub), indicated by a yellow flag, is attached to membrane proteins that are being endocytosed or transported from the TGN. This causes the proteins to be retained in Hrs- and clathrin-containing microdomains of the endosome membrane. Subsequently, the membrane proteins are sorted to intraluminal vesicles and targeted via MVEs for lysosomal degradation/processing. Note that a non-ubiquitinated membrane protein is not retained in Hrs–clathrin microdomains and is recycled to the plasma membrane. Clathrin coats are indicated in red. The size of the sorting endosome is overemphasised in order to show the sorting machinery in some detail. The area indicated by a rectangle is highlighted in (b). **(b)** Schematic presentation of the recently identified protein complexes that are involved in MVE sorting and formation. The lipid PI3P mediates the localisation of Hrs and its associated proteins Eps15, STAM and clathrin to endosomal membranes. This complex binds to a ubiquitinated receptor and retains it in the endosome membrane. Subsequently, the ubiquitinated receptor is delivered to ESCRT-I by interacting with Tsg101. The receptor is then relayed to ESCRT-II and transported into an intraluminal vesicle, which is formed through polymerisation of ESCRT-III complexes. The membrane association of several of these proteins is controlled by the AAA ATPase Vps4 (not shown). Before vesicle scission, the receptor is deubiquitinated. Arrows indicate the direction of the sorting process.

of energetically favourable tetrahelical coiled-coil bundles [53]. Since the pinching-off of a vesicle towards the endosome lumen might be regarded as a membrane fusion of the neck regions of the forming vesicle, a possible scenario could be that ESCRT-III complexes, through stable coiled-coil interactions, might function in inwards vesicle scission. Vps4 could then, by analogy with the AAA ATPase NSF (*N*-ethylmaleimide-sensitive factor) in membrane fusion, function to untangle coiled-coils to reactivate ESCRT complexes for further budding reactions.

Viruses hijack the multivesicular endosome formation and sorting machineries

As well as the inwards budding of endosomal membranes, another type of vesicle budding is also known to occur by direction from the cytosol – that of enveloped viruses that bud from the plasma membrane or into various organelles. Recent studies show that several enveloped RNA viruses reprogramme the MVE machinery for their budding [54•]. It has been known for some time that many small RNA viruses, such as HIV and Ebola virus, do not encode their own machineries for viral budding. Instead, they require a cellular function that is activated by the so-called 'late domain' of their membrane-associated structural proteins. Two proline-rich motifs in the late domain, P(S/T)AP and PPXY (where X is any amino acid), have been found to be essential for viral budding, and recent results link both these motifs to the MVE sorting machinery. The P(S/T)AP motif of HIV binds Tsg101, and in Tsg101-depleted lymphocytes HIV is unable to bud from the plasma membrane and instead forms stalks of unbudded virions [55]. HIV budding also requires mammalian Vps4, indicating that the ESCRT machinery is required for the scission of HIV particles from the plasma membrane [56•]. Thus, the virus appears to redirect the ESCRT complexes to the plasma membrane. It is interesting to note that in macrophages, HIV buds into MVEs [57•], so the virus is probably able also to use the ESCRT machinery at its normal location.

The other late-domain motif that is important for viral budding, PPXY, is a preferred binding motif for WW domains found in the Nedd4 family of HECT-domain-containing ubiquitin ligases. The recruitment of a ubiquitin ligase via the PPXY motif could perhaps explain the fact that the HIV gag protein is ubiquitinated [54•]. The role of this ubiquitination is not known, but *in vitro* studies have shown that the PSAP-motif-dependent binding of Tsg101 to HIV gag is strongly increased when the gag protein is also ubiquitinated [23•]. In a similar manner, VP40 of the Ebola virus, which is required for viral budding, binds Tsg101 via a P(S/T)AP motif and Nedd4 via a PPXY motif [58•,59•]. Intriguingly, both a P(S/T)AP and a PPXY motif can also be found in Hrs. This raises the possibility that Hrs could play a role in ESCRT and ubiquitin ligase recruitment to early endosomes and that viral proteins might function as Hrs mimics at the plasma membrane.

Viruses not only parasitise on the machinery for MVE formation — they may also usurp the MVE sorting machinery to subvert immune surveillance. An interesting example is provided by Kaposi's-sarcoma-associated herpes virus, whose gene product, K3, is a ubiquitin ligase that ubiquitinates newly synthesised MHC class I molecules, thus targeting them for lysosomal degradation in a Tsg101-dependent manner [60•]. Similarly, the related M153R product of myxomavirus ubiquitinates the T cell co-receptor CD4 and causes its lysosomal degradation [61•]. Viral tampering with the MVE sorting machinery could thus represent a widespread mechanism of immune evasion.

Conclusion and future perspectives

During the past year, we have witnessed great progress in our understanding of how MVEs are formed, and how proteins are sorted into them. The time is approaching when it might be possible to reconstitute MVE formation in the test tube from purified components. However, the mechanisms of MVE sorting and formation may be more complicated than we like to believe. For instance, mannose 6-phosphate receptors, which are known to recycle to the TGN after delivering their associated lysosomal enzymes to late MVEs, can be detected in intraluminal vesicles of MVEs [3•]. Does this mean that intraluminal vesicles can fuse back with the limiting MVE membranes, or could it be that what we perceive as intraluminal vesicles (by electron microscopy) in some cases simply represent deep invaginations? Furthermore, inhibition of PI3P formation on endosomes by microinjected antibodies against class III PtdIns 3-kinase appears to inhibit activated EGF receptors from entering into intraluminal vesicles of MVEs — but the receptors are nevertheless degraded as normal [44]. These findings suggest that the relationship between MVE sorting and receptor degradation could be more complex in mammalian cells than that described in yeast.

Aside from these complications, the following questions remain to be addressed: How do ubiquitin ligases recognise their specific targets? How can ubiquitin function to recruit different sorting machineries at the plasma membrane and at the endosome membrane? Does ubiquitin- and lipid-mediated sorting into MVEs represent two distinct pathways, or are they interlinked? By which mechanisms are non-ubiquitinated proteins sorted to intraluminal vesicles of MVEs? What is the role of the bilayered clathrin coat on endosomes? How do Hrs/Vps27 and the ESCRT complexes cooperate in protein sorting and MVE formation? How do inwards invaginations form? How do intraluminal vesicles pinch off from the limiting membrane? By which mechanisms do viruses reprogramme the MVE machineries? Hopefully, an equivalent review in a few years time can provide most of the answers.

Update

Recent studies have shed more light on the role of ubiquitin in protein sorting into MVEs. Because many mammalian receptor tyrosine kinases, such as the EGF receptor, migrate as smeared bands in SDS electrophoresis upon ubiquitination, it has been thought that these proteins become polyubiquitinated. This has been puzzling, since monoubiquitin mediates MVE sorting in yeast. Two papers now report that growth factor receptors, rather than being polyubiquitinated become monoubiquitinated at multiple lysine residues [63•,64•]. Thus, the principal function of monoubiquitin as an MVE sorting signal has been conserved from yeast to humans. The receptors for hepatocyte growth factor (Met) and insulin-like growth factor I provide new examples of growth factor receptors that are downregulated following ubiquitination, the former in a Hrs-dependent manner [65•,66]. A new ubiquitin-binding motif has been identified, the CUE domain [67]. This domain is found in several proteins of yeast and mammalian origin, and the CUE domain of a regulator of endocytic membrane fusion in yeast, Vps9, appears to negatively modulate the function of this protein in receptor trafficking [68]. This supports the idea that interactions with monoubiquitin not only might mediate protein sorting but also might serve to regulate protein activity.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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14. Hofmann K, Falquet L: **A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems**. *Trends Biochem Sci* 2001, **26**:347-350.

15. •• Lloyd TE, Atkinson R, Wu MN, Zhou Y, Pennetta G, Bellen HJ: **Hrs regulates endosome invagination and receptor tyrosine kinase signaling in *Drosophila***. *Cell* 2002, **108**:261-269.
Germline mutant embryos that lack full-length Hrs protein die early in embryogenesis, whereas zygotic mutants make it to the third-instar larvae/early pupae stage, probably owing to long-lived, maternally deposited Hrs. Hrs mutant larvae have enlarged endosomes, owing to an inability of endosomes to invaginate their limiting membrane, and hence a reduction in the number of multivesicular endosomes (MVEs) is observed. Hrs mutant embryos have increased activation and signalling from epidermal growth factor and Torso receptors, suggesting that trafficking into MVEs plays an important role in signal attenuation.

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This report shows that ubiquitinated proteins accumulate on epidermal growth factor (EGF)-containing endosomes in EGF-stimulated cells, together with mammalian class E proteins such as Tsg101 and hVps28. These proteins, in addition to Hrs, are further shown to interact with ubiquitin. Microinjection of anti-hVps28 antibodies or depletion of Tsg101 by small interfering RNA cause ubiquitinated proteins to accumulate on endosomes and retard EGF degradation.

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The interaction between ubiquitin- and ubiquitin-interacting motif (UIM)-containing proteins is characterised; Ile44 of ubiquitin is critical for its ability to bind UIMs. Whereas Epsin UIMs are important for internalisation of monoubiquitinated receptors from the plasma membrane, Vps27 UIMs are necessary to sort endocytic and biosynthetic cargo into the multivesicular endosome pathway.

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• Chen H, De Camilli P, Di Fiore PP: **A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins**. *Nature* 2002, **416**:451-455.
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• **Structure and functional interactions of the Tsg101 UEV domain**. *EMBO J* 2002, **21**:2397-2406.
This study continues the story of the role of Tsg101 in vacuolar protein sorting and virus budding recently published by the same group [55]. By resolving the structure of the Tsg101 UEV (ubiquitin-binding) domain and providing chemical shift mapping of the ubiquitin- and PTAP-binding sites, this report gives insight into how Tsg101 can act in these processes.

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• **Bilayered clathrin coats on endosomal vacuoles are involved in protein sorting toward lysosomes**. *Mol Biol Cell* 2002, **13**:1313-1328.
This report shows beautiful electron microscopy (EM) images of the endosomal clathrin/Hrs coat. The authors suggest a role for this coat in lysosomal protein sorting. Quantitative immuno-EM shows that the lysosomal targeted growth hormone receptor and the epidermal growth factor receptor are concentrated in the coat, whereas the recycling transferrin receptor is not.

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• *Curr Biol* 2002, **12**:R74-R78.
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The ESCRT-III complex is identified and characterised. It consists of two functionally distinct subcomplexes, Vps20–Snf7 and Vps24–Vps2, which are all class E Vps proteins. Vps24–Vps2 binds to the Vps20–Snf7 subcomplex, which is linked to membranes in part via the myristoyl group of Vps20. The authors present a model for protein sorting and multivesicular endosome formation that summarises the results from three ESCRT articles [24,50••,51•].

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After having resolved the structure of the Tsg101 UEV (ubiquitin-binding) domain (Pornillos *et al.* [2002] [23•]), the authors continue their investigation of the structure of this domain in complex with the HIV late-domain PTAP sequence. Interestingly, a similar motif (PSAP) is found in Hrs.

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By studying HIV-infected macrophages using electron microscopy, the authors show that virus particles accumulate in multivesicular compartments which are enriched in MHC class II and CD63. The data suggest that virus might access the lumen of this compartment by budding from the limiting membrane, similar to the formation of internal vesicles in multivesicular endosomes.

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This report shows that the V40 protein from Ebola virus can interact with human Nedd4 and Tsg101 *in vitro*. The WW domain 3 of Nedd4 is necessary and sufficient for binding to the PPXY motif of V40, whereas the UEV domain of Tsg101 is sufficient to bind the PTAP domain of the virus protein.

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This study gives another intriguing example of how viruses can induce endocytic downregulation of host proteins, thereby evading the cellular immune response.

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This paper and Mosesson *et al.* (2003) [64•] show evidence that receptors for epidermal growth factor and platelet-derived growth factor become multiply monoubiquitinated (and not polyubiquitinated) upon ligand binding. A single attached ubiquitin appears to be sufficient for endocytosis and lysosomal degradation.

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