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Molecular mechanisms of late endosome morphology, identity and sorting

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Recent studies using electron microscopy, protein crystallography, classic biochemistry and novel live-cell imaging have provided numerous insights into the endocytic pathway, describing a dynamic system in which compartment morphology, molecular identity and the mechanics of cargo sorting are intimately connected. Current evidence supports a model of maturation in which the lipids, cargo proteins and Rab population at the endosome determine its competence to perform the functions of late endosomes, including the sorting of cargoes into lumenal vesicles and fusion with lysosomes.

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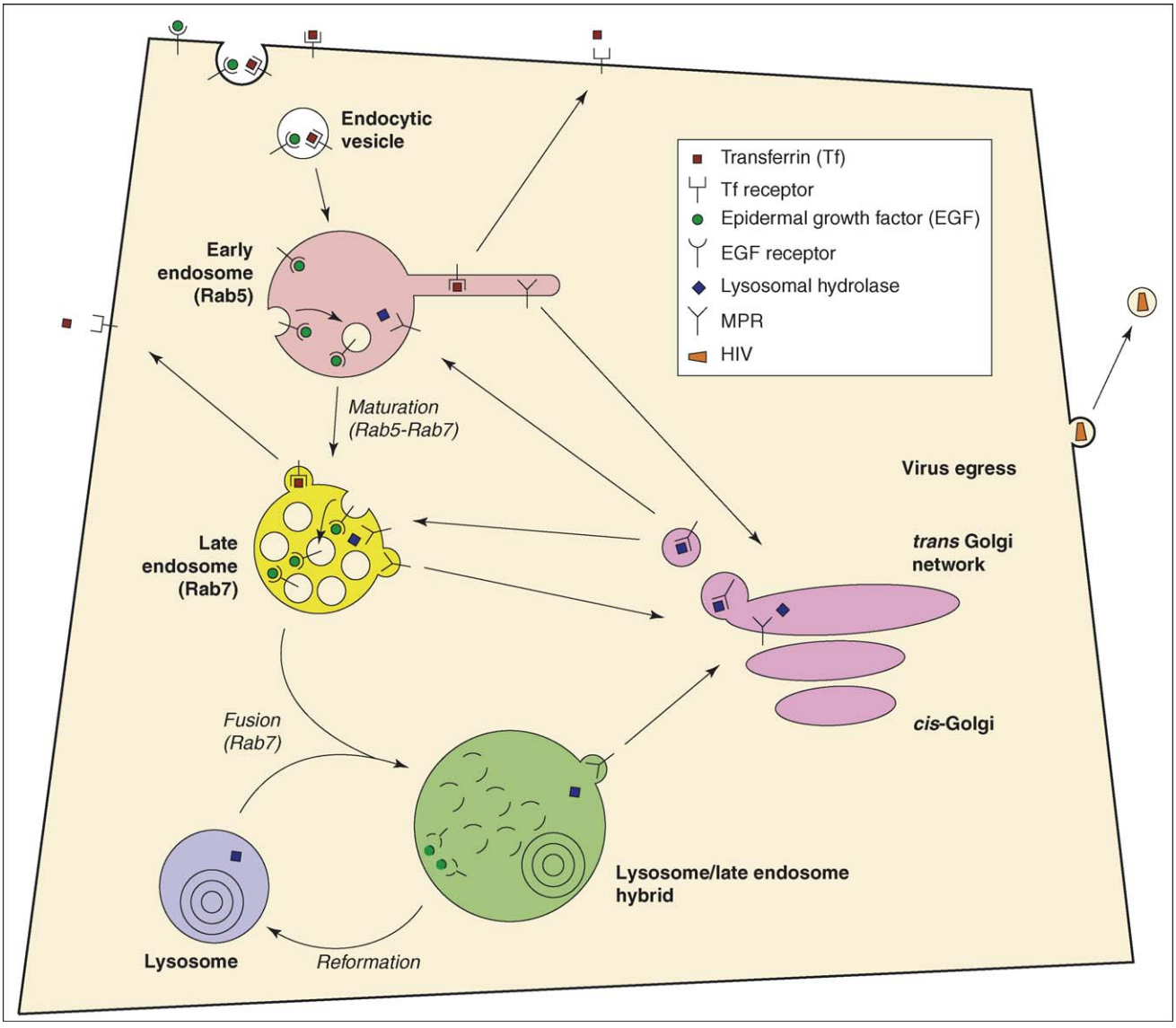
Introduction

The endocytic pathway is a highly dynamic system that coordinates multiple trafficking routes (Figure 1). The lysosome is generally considered the end point of the endocytic pathway, and most of the biosynthetic and endocytic proteins targeted to this degradative compartment must pass through late endosomes. The biogenesis of late endosomes occurs by a process of maturation from vacuolar elements of early endosomes, during which time regions of the perimeter endosomal membrane bud toward the compartment lumen and pinch off to form vesicles. As a consequence of this distinctive morphology, late endosomes are commonly referred to as multivesicular bodies (MVBs). Membrane proteins destined for degradation within the lysosome lumen are sorted into lumenal MVB vesicles through the concerted action of the conserved class E Vps machinery. In mammals, the process is initiated when an early component of this machinery, Hrs, recruits clathrin to form an atypical coat. At the same time, membrane proteins bound for the plasma membrane or *trans*-Golgi network are excluded from this coat and recycled away from late endosomes, while other proteins that remain in the late endosome perimeter membrane are eventually delivered to the lysosomal membrane following late endosome–lysosome fusion (Figure 1) [1–3]. A similar sequence of events seems to occur in yeast except that a clathrin coat has not been identified. This review examines recent insights derived from a combination of conventional and novel techniques that have begun to address how late endosomes are regulated. Specifically, we discuss how lipids and Rab GTPases determine the morphology and identity of late endosomes, and we summarize advances in understanding the molecular basis of ubiquitin-dependent cargo protein sorting derived from new structural studies of the late endosomal sorting machinery. We consider the possibility that lumenal vesicle formation is coordinated with sorting to those vesicles and other late endosome functions. Finally, we examine recent insights into the relationship between the yeast and mammalian endocytic pathways.

Late endosome morphology and identity

In vitro work has suggested how lipids may promote the multivesicular morphology characteristic of late endosomes. The budding of vesicles into the lumen of synthetic liposomes with a phospholipid content similar to that of late endosomes was found to be dependent upon 2,2′-dioleoyl lysobisphosphatidic acid (LBPA), a structural isomer of phosphatidylglycerol that is enriched in a subset of mammalian late endosomal membranes [4••,5]. The ability of LBPA to induce membrane invagination is consistent with the fact that this lipid has an inverted cone shape and a small head group, a structure that prefers curved membranes. However, LBPA also dramatically enhances the fusion of synthetic liposomes with one another [6], which suggests that LBPA may also promote 'back-fusion' of lumenal membranes with the perimeter of late endosomes. Indeed, this type of retrograde fusion event, which is typical of MVBs that have a non-degradative function [7], delivers vesicular stomatitis virus [8] and anthrax lethal factor [9] into the cytosol of infected cells and is inhibited when LBPA function is disrupted [8]. However, it seems unlikely that LBPA provides a generic inward vesicle-forming mechanism as LBPA has not been detected in yeast. Furthermore, LBPA is restricted to only a subpopulation of multivesicular late endosomes. Interestingly, the LBPA-containing MVBs

Figure 1



The place of late endosomes in the mammalian endocytic pathway. Late endosome maturation from early endosomes involves concurrent exchange of Rab7 for Rab5, removal of non-lysosomal cargo (e.g. transferrin receptors and MPRs), formation of, and incorporation of MVB cargoes into, lumenal vesicles (e.g. ligand-bound EGF receptors). Mature late endosomes fuse with lysosomes, which allows lumenal cargo degradation, and in mammals leads to a hybrid compartment from which lysosomes reform [1]. The model shows direct fusion but transient interactions also occur [16•]. Lumenal vesicle formation and budding of HIV from the cell surface are topologically equivalent and involve the class E Vps machinery. Sorting into MVB vesicles by this machinery includes recruitment of clathrin to endosomes to form a coat that excludes recycling cargoes such as transferrin receptors. No such coat has been identified in yeast. MPR, mannose-6-phosphate receptor; HIV, human immunodeficiency virus.

are distinct from the MVBs at which ubiquitinated membrane proteins are sorted [5].

The discovery of these distinct endosomal populations represents a new complication in understanding the relationship between early and late endosomes in mammals. Similarly, mannose-6-phosphate receptors (MPRs) and an alternative acid hydrolase receptor transit via late endosomes but are located in different subpopulations [10]. While it is not yet certain whether a single early endosome can give rise to different types of late endosomes, these data might resolve the confusion derived from studies indicating that late endosomal lumenal membranes are associated with molecules targeted for lysosomal degradation (e.g. EGF) as well as molecules not normally degraded by lysosomes (e.g. MPR

and LBPA) if the membranes are located in different MVBs.

The budding of MVB vesicles marks to some extent the biogenesis of late endosomes, which arise from the progressive maturation of early endosomes (for example [2]). Rab GTPases have historically served a major role in determining the molecular identities of endosomal compartments, including early endosomes, which contain Rab5, and late endosomes, which contain Rab7 [11]. By simultaneously tracking the distributions of Rab5 and Rab7 over time using live-cell fluorescence microscopy, individual compartments in mammalian cells were discovered to undergo an exchange in which Rab5 is replaced by Rab7 [12••]. Independently, another study found that Rab7-positive membrane domains form on Rab5-positive early endosomes and then bud away [13]. Both studies are consistent with the formation of Rab7-positive late endosomes from elements of early endosomes (Figure 1). Although additional maturation steps might be involved, it is noteworthy that Rab7 is associated with the competence of late endosomes to fuse with lysosomes [14] and that disruption of Rab7 function causes accumulation of endocytosed epidermal growth factor (EGF) in compartments that have the density and protein content of late endosomes [15]. Refining our understanding of these later events, another live mammalian cell study shows that content exchange between late endosomes and lysosomes occurs both by fusion and by transient 'kiss-and-run' interactions [16•]. Again, no vesicular traffic was observed between these compartments.

Ubiquitin-dependent sorting of MVB cargoes

The concentration of cargo proteins into lumenal MVB vesicles has been a primary focus of research during the past few years. Integral membrane proteins are selected as MVB cargoes by virtue of post-translational attachment of either a single ubiquitin or a short chain of two to three ubiquitin subunits to their cytosolic domains. This ubiquitin modification is recognized by ubiquitin-binding domains present within subunits of ESCRT (endosomal sorting complex required for transport)-I and ESCRT-II as well as a complex sometimes referred to as ESCRT-0 (Table 1). The ESCRTs are cytosolic protein complexes that were originally characterized as members of the 'Class E' subset of *VPS* gene products in yeast but appear to be conserved throughout eukaryotes [17]. The dominant hypothesis of ESCRT function entails the transfer of cargoes first from ESCRT-0 to ESCRT-I and then from ESCRT-I to ESCRT-II (reviewed in [18]), and several recent structural and functional studies have reinforced the attractiveness of this model. Crystal structures obtained for the second UIM domain of yeast Vps27 [19], the UEV domains of yeast Vps23 [20] and mammalian TSG101 [21], and the solution structure of the NZF domain of yeast Vps36 [22] have been accompanied by

Table 1

Selected proteins required for MVB cargo protein sorting

Yeast	Mammal	Complex	Features/motifs	Binds	Structure
Vps27	Hrs	ESCRT-0	FYVE, UIM, VHS	PI(3)P, Ub	UIM (crys/Sc)
Hse1	STAM1, 2	ESCRT-0	UIM, SH3	Ub	
Vps23	TSG101	ESCRT-I	UEV, CC	Ub	UEV/Ub (crys/mam, crys/Sc)
Vps37	VPS37A,B,C,D	ESCRT-I	CC		
Vps28	VPS28	ESCRT-I			
Vps36	EAP45	ESCRT-II	NZF (Sc)/GLUE (mam)	Ub, PI(3,4,5)P3 (mam)	ESCRT-II complex (crys/Sc)
Vps22	EAP30	ESCRT-II	CC		NZF/Ub (sol/Sc)
Vps25	EAP20	ESCRT-II			
Snf7	CHMP4A,B,C	ESCRT-III	Charged, CC	PI(3)P (mam)	
Vps20	CHMP6	ESCRT-III	Charged, CC		
Vps2	CHMP2A,B	ESCRT-III	Charged, CC		
Vps24	CHMP3	ESCRT-III	Charged, CC	PI(3,5)P2	
Bro1	Alix/AIP1	Unknown	Bro1, CC, PRD	LBPA	Bro1 domain (crys/Sc)
Vta1	LIP5	w/Vps60	CC		
Vps60	CHMP5	w/Vta1	Charged, CC		
Did2	CHMP1A, B	Unknown	Charged, CC		
Vps4	VPS4A,B/SKD1	Homomultimer	MIT, AAA		MIT (sol/mam) Monomer (crys/mam)

ESCRT, endosomal sorting complex required for transport; FYVE, 'Fab1, YOTB, Vac1, EEA1'; UIM, ubiquitin-interacting motif; VHS, 'Vps27, Hrs, STAM'; Crys, crystal structure; Sc, *Saccharomyces cerevisiae*; SH3, Src-homology-3; Ub, ubiquitin; PI(3)P, phosphatidylinositol 3-phosphate; UEV, ubiquitin E2 variant; CC, coiled-coil; Mam, mammalian; NZF, Npl4 zinc finger; Sol, solution structure; GLUE, GRAM-like ubiquitin-binding in Eap45; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-triphosphate; Myr, myristoylation; $PI(3,5)P_2$, phosphatidylinositol 3,5-bisphosphate; Bro1, Bro1 domain; PRD, proline-rich domain; LBPA, lysobisphosphatidic acid; MIT, microtubule interaction and transport domain; AAA, ATPase associated with a variety of cellular activities.

mutational analyses demonstrating that disruption of ubiquitin-binding by these domains impairs sorting of MVB cargoes. These structural studies also revealed that ESCRT-0, -I and -II recognize the same isoleucine-44 hydrophobic patch on the ubiquitin surface, further suggesting sequential cargo recognition rather than simultaneous binding interactions. A major hole in this sequential 'ubiquitin hand-off' model of ESCRT function had been the apparent lack of a ubiquitin-binding domain in the mammalian ESCRT-II complex, but this issue was resolved by discovery of the ubiquitin-binding GLUE domain in the Vps36 ortholog, EAP45 [23•]. Nevertheless, it remains to be tested experimentally whether directional transfer of ubiquitinated cargoes from one ESCRT complex to another occurs.

The ESCRT-III complex in both yeast and mammalian cells lacks ubiquitin-binding domains, but its assembly on late endosomal membranes is essential for the recruitment of other Class E Vps proteins that function downstream of cargo recognition (Table 1; [24]). For instance, the yeast Bro1 protein is recruited by the Snf7 subunit of ESCRT-III and, in turn, mediates the recruitment of Doa4, a ubiquitin thiolesterase that removes ubiquitin from MVB cargoes [25•]. Analysis of a crystal structure of the conserved amino-terminal domain of Bro1 revealed a hydrophobic patch of amino acids that mediates binding to Snf7 [26], but the site on Snf7 involved in this interaction remains unknown.

ESCRT-III also is required for recruitment of Vps4, an AAA-type ATPase that catalyzes dissociation of ESCRTs and the rest of the class E Vps machinery from late endosomal membranes. The solution structure of the MIT domain of human Vps4b [27] closely resembles the solution structure of the human Vps4a MIT domain [28], providing a picture of how this region of Vps4 interacts with ESCRT-III subunits and possibly other class E Vps protein substrates. Specifically, three helices in the MIT domain pack together to form an incomplete tetratricopeptide repeat (TPR), leaving a groove between two helices that can be occupied by ESCRT-III proteins to complete the TPR structure. Accordingly, the C-terminal ends of human CHMP1, CHMP2, and CHMP3 each contain an amphipathic helix capable of binding the MIT domain (WI Sundquist *et al.*, personal communication). The crystal structure of monomeric human Vps4b revealed that the catalytic domain closely resembles that of another AAA-type ATPase, p97, but contains a novel β domain for the binding of LIP5 [29•]. Studies in yeast demonstrate the regulatory significance of this interaction, as Vta1 (the yeast ortholog of LIP5) stimulates oligomerization of, and ATP hydrolysis by, Vps4 [30]. Moreover, a predicted structure for multimeric Vps4b suggests a central pore through which class E protein substrates may be 'pulled' in order to facilitate their dissociation from the endosomal membrane [29•].

Coordinating MVB cargo sorting with other late endosome functions

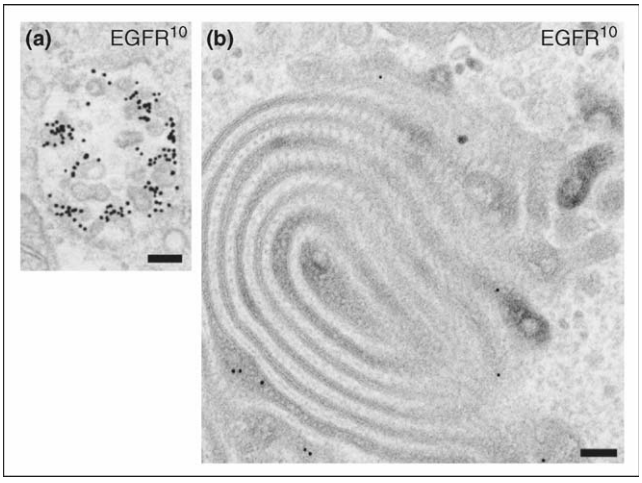
The functions of ESCRT complexes and other Class E Vps components appear to be linked to the mechanism of MVB vesicle formation. This connection seems particularly apparent in light of the role that the class E Vps machinery has in the budding of enveloped viruses from infected cells (reviewed in Morita and Sundquist, 2004 [31]), a process that is topologically equivalent to MVB vesicle budding (Figure 1). Moreover, in yeast, mutation of any class E *VPS* gene disrupts the multivesicular morphology of late endosomes, resulting in stacked, flattened cisternae, commonly called 'class E compartments' (e.g. [32]). The absence of lumenal vesicles within class E compartments suggests that sorting of cargoes is coordinated with vesicle formation.

However, recent work has shown how sorting can be uncoupled from vesicle formation [33]. Overexpression of an Hrs mutant in which the ubiquitin-binding UIM domain has been deleted significantly reduces the precentage of EGF receptors sorted into lumenal vesicles without affecting the overall number of vesicles. In contrast, more deleterious mutations in Hrs significantly reduce the number of lumenal vesicles. It therefore seems that Hrs may have separable functions that are required for cargo sorting versus lumenal vesicle formation, although the molecular basis of Hrs function in vesicle formation is not known.

Recently, structures that resemble class E compartments have also been induced in mammalian cells in which ESCRT-I function is disrupted by siRNA-mediated depletion of TSG101 (Figure 2, [34••]). Such multicisternal structures are not, therefore, unique to yeast. In addition to defects in lumenal vesicle formation, recycling pathways are disrupted following TSG101 depletion, resulting in accumulation of mannose-6-phosphate receptors and transferrin receptors in class E compartments [34••]. This apparent block in retrograde transport is associated with the sustained presence of early endosome markers in class E compartments and the inability of these aberrant structures to fuse with lysosomes. These effects suggest that the class E Vps machinery normally coordinates endosome maturation with MVB vesicle formation and that multicisternal endosomes are a consequence of disruption to this coordination. However, overexpression of an ATPase-defective Vps4 allele in mammalian cells also impairs lumenal vesicle formation but, unlike the effect observed upon TSG101 depletion, leads to enlarged vacuolar endosomes that appear to undergo maturation and to fuse with lysosomes [35]. Thus, endosomal maturation is not necessarily coordinated with lumenal vesicle formation under the direction of class E Vps protein functions.

Finally, recent evidence has revealed that cargoes themselves can influence late endosome biogenesis.

Figure 2



The mammalian multicisternal 'class E' compartment. A normal MVB **(a)** compared to a multicisternal class E endosome **(b)** in mammalian cells. 10 nm gold particles indicate EGF receptors which exit normal MVBs and arrive in lysosomes within 60 minutes of internalisation, but remain in class E endosomes for at least 3 hours without reaching lysosomes. (a) reproduced from [2] by copyright permission of The Rockefeller University Press. (b) Image is unpublished data from the Doyotte *et al*. 2005 [34••] study (electron dense content: crosslinked horseradish peroxidase-conjugated transferrin). Scale bars represent 100 nm. EGFR, EGF receptor.

Stimulation of cells with EGF increases the number of MVBs as well as the number of lumenal vesicles within each compartment [36••]. The latter activity requires phosphorylated annexin I, a major EGF receptor tyrosine kinase substrate [36••]. The molecular function of annexin I in MVB biogenesis has yet to be determined, but its ability to respond to increased endocytic cargo flux is not restricted to EGF-stimulated pathways. A similar mechanism seems to occur in anergic T-cells, where downregulation of T-cell receptors is accompanied by increased synthesis of annexin I and TSG101 [37,38].

Yeast versus mammalian late endosomes

The high degree of conservation in the class E Vps sorting machinery and the finding that disruption to this machinery can cause similar morphological and trafficking defects in yeast and mammals raises the question of what the relationship is between the endocytic pathways of these organisms. In particular, the early endocytic pathway has had limited characterization in yeast [39]. For example, no Hrs/clathrin coat [3] has been identified in yeast. Furthermore, the early endocytic MVB precursors (tubular-vacuolar structures with occasional lumenal vesicles) on which this coat is found are clearly visible in mammalian cells but have not been observed in yeast. Interestingly, the aberrant endosomes that replace MVBs in most studies of mammalian cells in which class E proteins are disrupted more closely resemble these vacuolar MVB precursors [35,40]. This is in contrast to yeast studies where aberrant endosomes take the form of multicisternal structures [39,41].

Hrs and TSG101 may in fact play distinct roles in mammals [40]. TSG101 is required for the formation of stable MVBs, since depletion of TSG101 causes formation of multicisternal endosomes. In contrast, Hrs depletion only affects lumenal vesicle formation and endosome homotypic fusion; late endosomes can still form and fuse with lysosomes. Since disruption to each of the yeast TSG101 and Hrs orthologues (or their complexes) causes multicisternal structures to form [32,39], the mechanisms for stable MVB formation may be more robust in mammals. This may be due to redundancy in the class E proteins in mammals (Table 1). Perhaps lumenal vesicle formation and endosome maturation are less tightly coupled in mammalian cells, allowing the system to be more flexible to accommodate the more complex trafficking activities of higher organisms [17].

There are also differences in retrieval mechanisms from the late endocytic pathway. In yeast, late endosomes fuse with the large hydrolytic vacuole, while in mammals there exists a dynamic system of hydrolytic lysosomes and late endosome/lysosome hybrids, both of which can fuse with late endosomes [1]. The recently characterized 'retromer' complex plays a role in retrograde transport from endosomes in both systems [42–44], but a Rab9-dependent retrieval pathway restricted to late endosome/lysosome hybrids only appears to exist in mammals [45].

Concluding remarks

The accumulation of both early and late endocytic cargoes in class E compartments signifies that the molecular identity of these aberrant structures is disrupted. Therefore, it would be of interest to determine which Rabs are associated with class E compartments, given the recent discoveries of Rab exchange during endosomal maturation [12••,13]. The clinical importance of this issue is illustrated by various reports of disruption to phagosome maturation by intracellular pathogens (including the prevention of Rab7 acquisition [46]). Moreover, the recent characterization of machineries involved in recycling from late endosomes [43–45] provides a framework for investigating how lumenal vesicle formation is coordinated with recycling pathways to facilitate the endosomal maturation process.

Lastly, it is worth noting that the advances in our structural and mechanistic understanding of ESCRT-0, -I and -II as well as Bro1 and Vps4 stand in stark contrast to what is known about ESCRT-III assembly. Since the initial description of its core components [24], there continues to be no purified ESCRT-III complex, no set stoichiometry and no structural data beyond computational sequence analysis. This dearth of information is bothersome in light of predictions that ESCRT-III oligomerization functions

in the concentration of cargoes and that ESCRT-III dissociation is required for MVB vesicles to pinch off from the perimeter late endosomal membrane. Current investigations might provide insight into whether these models regarding the role of ESCRT-III hold true.

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