

A concentric circle model of multivesicular body cargo sorting

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Targeting of ubiquitylated transmembrane proteins into luminal vesicles of endosomal multivesicular bodies (MVBs) depends on their recognition by endosomal sorting complexes required for transport (ESCRTs), which are also required for MVB vesicle formation. The model originally proposed for how ESCRTs function succinctly summarizes much of the protein–protein interaction and genetic data but oversimplifies the coordination of cargo recognition and cannot explain why ESCRTs are required for the budding of MVB vesicles. Recent structural and functional studies of ESCRT complexes suggest an alternative model that might direct the next series of breakthroughs in understanding protein sorting through the MVB pathway.

Keywords: endosome; ESCRT; MVB; ubiquitin; vesicle

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Introduction

Membrane transport pathways from both the plasma membrane and Golgi intersect at endosomes. As endosomes mature into multivesicular bodies (MVBs), some transmembrane proteins are incorporated into vesicles that bud into the lumen (Fig 1). Most of these transmembrane proteins are marked as cargo molecules of luminal MVB vesicles by a single ubiquitin (Ub) or a short chain of two to three Ub subunits that are added to their cytosolic domains after translation. When the limiting (outer) membrane of a MVB fuses with the lysosome in animal cells or with the vacuole in yeast, MVB vesicles and their protein contents become vulnerable to degradation (reviewed by Babst, 2005; Russell *et al.*, 2006; Hurley & Emr, 2006).

Both the biogenesis of MVB vesicles and the recognition of ubiquitylated transmembrane proteins as MVB vesicle cargoes depend on a set of cytosolic proteins known as the ‘class E’ vacuolar protein sorting (Vps) proteins (Table 1). Class E Vps proteins were originally identified in genetic screens for defects in the sorting of enzymes to the yeast vacuole (Raymond *et al.*, 1992). Every eukaryotic organism for which a complete genome is available has orthologues of class E Vps proteins, and studies in various

model systems indicate that their specific functions are conserved. Therefore, for simplicity, only the names of yeast class E Vps proteins are used below unless details of certain class E Vps proteins outside yeast deserve distinction.

Many class E Vps proteins assemble to form distinct complexes that have been termed endosomal sorting complexes required for transport (ESCRTs; see Table 1). ESCRTs associate transiently with the endosomal membrane, and their dissociation from endosomes depends on another class E Vps protein, Vps4, which is a member of the AAA family of ATPases. The first model to depict ESCRT function (Babst *et al.*, 2002a) was a landmark in the study of MVB sorting as it synthesized a wide array of genetic and biochemical data into a readily accessible picture in which the activities of ESCRTs resembled an industrial conveyor belt. Virtually all of the studies on ESCRTs that followed have been interpreted in this context, leading to the current ‘conveyor belt model’ of ESCRT function (Fig 2; reviewed by Hurley & Emr, 2006). According to this model, MVB sorting begins with endosomal recruitment of the Vps27–Hse1 (has symptoms of class E mutants 1) complex (also known as ESCRT-0) through binding of Vps27 to phosphatidylinositol 3-phosphate (PI(3)P), which is enriched in the cytosolic leaflet of endosomal membranes. ESCRT-0 binds to ubiquitylated cargoes by virtue of Ub-interacting motifs (UIMs) in both Vps27 and Hse1, thereby establishing the molecular basis for the initial recognition of MVB cargoes. Vps27 mediates the recruitment of ESCRT-I to endosomal membranes by binding to Vps23 in the ESCRT-I complex. Similarly to ESCRT-0, ESCRT-I binds to ubiquitylated cargoes, in this case through the Ub E2 variant (UEV) domain of Vps23. ESCRT-II binds to ubiquitylated cargoes through the Npl4 zinc finger (NZF) domain in yeast Vps36 or the GRAM-like Ub binding in Eap45 (GLUE) domain in the Vps36 orthologue in mammalian cells. Overexpression of the entire ESCRT-II complex in yeast suppresses the defect in MVB cargo sorting that is caused by disruption of ESCRT-I, suggesting that ESCRT-II functions downstream from ESCRT-I.

The conveyor belt model continues with the recruitment of ESCRT-III, which comprises four homologous proteins—Vps20, Vps2, Vps24 and Snf7 (sucrose non-fermenting 7)—which are thought to associate tightly with the endosomal membrane. Vps20 is myristoylated at its amino terminus, which presumably inserts into the cytosolic leaflet of the endosomal membrane. However, efficient localization of ESCRT-III to endosomes also depends on the binding of Vps20 to the Vps25 subunit of ESCRT-II. In contrast

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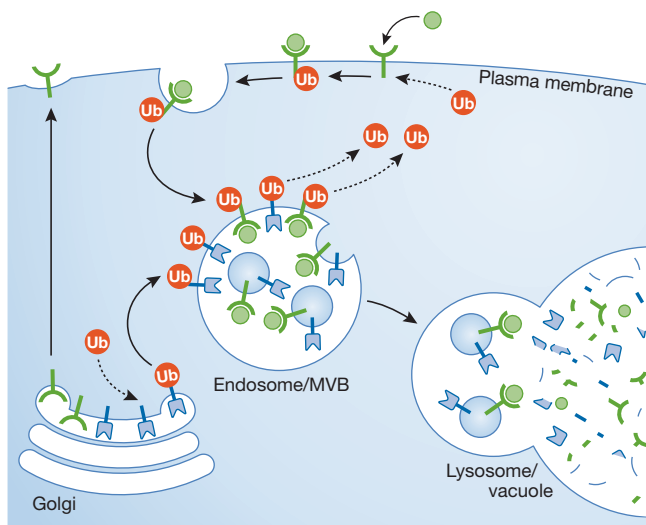


Fig 1 | The multivesicular body pathway. Endocytic and biosynthetic transmembrane cargoes are ubiquitylated to facilitate their entry into luminal vesicles of MVBs. Monoubiquitylation is the most common form of modification of MVB cargoes, although polyubiquitylation and multiple monoubiquitylation are also known to occur. When the MVB fuses with the lysosome/vacuole, its luminal contents are degraded. MVB, multivesicular body; Ub, ubiquitin.

to ESCRT-0, -I and -II, none of the ESCRT-III proteins binds to ubiquitylated cargoes and, rather than assemble into a discrete complex of uniform molecular weight, ESCRT-III is presumed to form an oligomeric array on the endosomal membrane (Babst *et al*, 2002a). Although a specific molecular mechanism has yet to be attributed to ESCRT-III, it is required for the recruitment of class E Vps proteins that are thought to terminate the conveyor belt model of ESCRT function. These include BCK1-like resistance to osmotic shock (Bro1), which mediates the recruitment of the Ub hydrolase degradation of alpha 4 (Doa4) that deubiquitylates MVB cargoes. In addition, ESCRT-III is required for the recruitment of Vps4, the ATPase that catalyses the dissociation of all ESCRTs from endosomal membranes (Babst *et al*, 2002a).

The challenge of determining how multiple cytosolic protein complexes cooperate to select cargo proteins and to promote endosomal membrane invagination should not be understated. Consequently, the conveyor belt model has been useful for depicting many of the known protein–protein interactions. Indeed, the graphic appeal of this model has reached such a level of popularity that it is featured in textbooks at the college and university level; a crucial step in the codification of dogma. However, it is our belief that sufficient data are now available to make an informed critique of the conveyor belt model and its predictions. At the onset, we wish to acknowledge our emphasis on the yeast model in our discussion. Obviously, considerable insights have been obtained through studies in metazoan models, but we believe a coherent, testable model emerges more readily from the simpler system, especially considering that many ESCRT proteins in mammalian cells perform functions apparently unrelated to endosomal transport (reviewed by Slagsvold *et al*, 2006). We hope that our presentation of an alternative model for MVB sorting persuades the reader that other interpretations of the available data are still possible.

Table 1 | Class E vacuolar protein sorting (Vps) and other selected proteins required for multivesicular body cargo sorting

Complex	Yeast	Mammal	Binds
ESCRT-0			
	Vps27	Hrs	Ub, PI(3)P, Vps23
	Hse1	STAM1, 2	Ub, Rsp5
ESCRT-I			
	Vps23	TSG101	Ub, Vps27
	Vps28	VPS28	Vps20, Vps36
	Vps37	VPS37A, B, C, D	—
	Mvb12	—	—
ESCRT-II			
	Vps36	EAP45	Ub, PI(3)P, Vps28
	Vps22	EAP30	—
	Vps25	EAP20	Vps20
ESCRT-III			
	Snf7	CHMP4A, B, C	Bro1, Vps4, PI(3)P
	Vps20	CHMP6	Vps28, Vps25, Vps4
	Vps2	CHMP2A, B	—
	Vps24	CHMP3	Did2, PI(3,5)P ₂
Other			
	Bro1	Alix	Doa4, Snf7
	Rsp5	Nedd4*	Hse1
	Doa4	AMSH*, UBPY*	Ub, Bro1
	Did2	CHMP1A, B	Vps4, Vps24, Vta1
	Vps60	CHMP5	Vta1
	Vta1	LIP5	Vps4, Did2, Vps60
	Vps4	VPS4A, B/SKD1	Did2, Snf7, Vps20, Vta1

*Apparent functional analogue. Alix, ALG2-interacting protein; AMSH, associated molecule with SH3 domain of STAM; Bro, BCK1-like resistance to osmotic shock; CHMP, charged multivesicular body protein; Did, Doa4-independent degradation; Doa, degradation of alpha; EAP, ELL-associated protein; ESCRT, endosomal sorting complex required for transport; Hrs, hepatocyte growth factor-regulated tyrosine-kinase substrate; Hse, has symptoms of class E mutants; LIP, LYST-interacting protein; Mvb, multivesicular body; Nedd, neuronal precursor cell-expressed developmentally downregulated; PI(3)P, phosphatidylinositol 3-phosphate; PI(3,5)P₂, phosphatidylinositol-3,5-bisphosphate; Rsp, reverses spt phenotype; SKD, suppressor of K⁺ transport growth defect; Snf, sucrose non-fermenting; STAM, signal transducing adaptor molecule; TSG, tumour suppressor gene; Ub, ubiquitin; UBPY, ubiquitin-specific protease Y; VPS, vacuolar protein sorting; Vta, Vps-twenty-associated.

Examining the conveyor belt model

The conveyor belt model assumes that the order of recruitment of ESCRT complexes indicates the order of activity, which results in several hypotheses: ESCRT-0, -I, -II and -III activities are sequential and linear; MVB cargo proteins are passed from one ESCRT complex to another; ‘earlier’ components such as ESCRT-0 and -I are spatially and temporally further away from forming MVB vesicles than are ‘later’ components such as ESCRT-III; and ESCRT-III is proximal to the forming vesicle, with dissociation of ESCRTs by Vps4 being the conclusion of vesicle formation. Although much of the evidence is consistent with these hypotheses, some data clearly disagree.

Are ESCRT activities sequential and linear? Yeast two-hybrid analysis revealed a triangular network between ESCRT-I, -II and -III, questioning the strict linearity of interactions (Bowers *et al*, 2004).

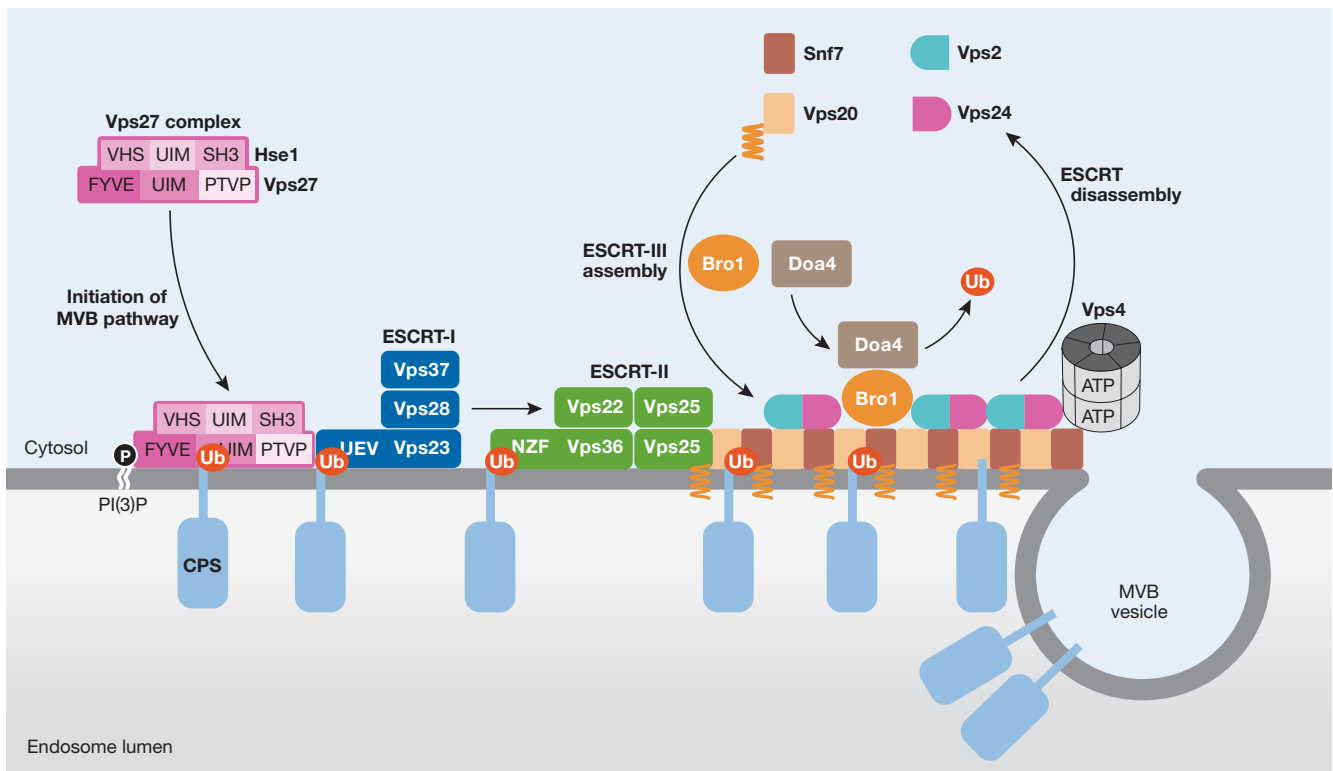


Fig 2 | The conveyor belt model of ESCRT function (reproduced from Hurley & Emr, 2006). According to this model, ESCRT complexes are recruited sequentially to the endosome and recognize ubiquitylated transmembrane proteins, passing cargo from one complex to the next to facilitate sorting to MVB vesicles. Deubiquitylation of cargoes by the Ub hydrolase Doa4 and disassembly of ESCRTs by the ATPase Vps4 precede invagination. See main text and Hurley & Emr (2006) for further description. Bro, BCK1-like resistance to osmotic shock; CPS, carboxypeptidase S; Doa, degradation of alpha; ESCRT, endosomal sorting complex required for transport; FYVE, ‘Fab1, YOTB, Vac1, EEA1’; Hse, has symptoms of class E mutants; MVB, multivesicular body; NZF, Npl4 zinc finger; PI(3)P, phosphatidylinositol 3-phosphate; PTPV, proline threonine valine proline; SH3, Src-homology 3; Snf, sucrose non-fermenting; STAM, signal transducing adaptor molecule; Ub, ubiquitin; UEV, Ub E2 variant; UIM, Ub-interacting motif; VHS, Vps27, Hrs, STAM; Vps, vacuolar protein sorting.

Direct binding between ESCRT-I (Vps28) and ESCRT-III (Vps20) was recently confirmed (Pineda-Molina *et al*, 2006) and showed that although ESCRT-II might mediate an interaction between ESCRT-I and -III, it is not required. Although this direct link has not been observed in mammalian cells, the interaction between ESCRT-I and -III is bridged by Alix, the orthologue of yeast Bro1 (Strack *et al*, 2003). It is important to note that both ESCRT-II and -III bind to Vps28 of ESCRT-I, and both ESCRT-I and -II bind to Vps20 of ESCRT-III. Therefore, an ESCRT triumvirate cannot be assumed to exist unless simultaneous binding occurs at each of these hubs.

Genetic evidence also questions the strict linear relationship between ESCRTs. Although ESCRT-II seems to be essential for MVB function in *Drosophila melanogaster* (Thompson *et al*, 2005), small interfering RNA-mediated knockdown of ESCRT-II in HeLa cells has no effect on the rate of epidermal growth factor (EGF) degradation (Bowers *et al*, 2005), suggesting that ESCRT-II is not required in all circumstances. Furthermore, in yeast, expression of a mutant Vps27 (ESCRT-0) that cannot bind to Vps23 (ESCRT-I) causes only a partial defect in cargo sorting (Bilodeau *et al*, 2003). Therefore, we conclude that although ESCRTs tend to act in sequence, the system is sufficiently flexible to allow some degree of compensation when this sequence is disrupted.

Are ubiquitylated cargoes sequentially transferred from ESCRT-0 to -I to -II? The sequential transfer hypothesis has become pervasive despite not having any data to support it. The strongest evidence for the functional importance of ubiquitylated cargoes interacting with several ESCRTs came from the identification of point mutations in Ub that allow it to interact with only Vps27 (ESCRT-0) or with only Vps23 (ESCRT-I). By expressing each mutant version of Ub in frame with the cytosolic domain of a transmembrane protein not normally sorted into luminal MVB vesicles, binding to both Vps27 and Vps23 was found to be required for entry into the MVB pathway (Bilodeau *et al*, 2003). However, expression of a Vps27 Ub-binding mutant produces no defect in the sorting of the MVB cargo Ste3 (Bilodeau *et al*, 2002), indicating that binding to both Vps27 and Vps23 is not a general requirement for MVB sorting. By contrast, mutations in the NZF domain in yeast Vps36 (ESCRT-II), which prevent Ub binding *in vitro*, also result in the accumulation of endosomal membranes (Alam *et al*, 2004), a phenotype that typically indicates MVB dysfunction. Therefore, it is unclear whether Ub recognition by ESCRT-II is required for cargo sorting or, instead, whether the mutations cause a broader defect in ESCRT-II beyond its role in cargo recognition (see Supplementary information online, (Un)importance of cargo selection).

Studies of an unusual MVB cargo in yeast, Sna3, are also inconsistent with the necessity of sequential transfer of cargoes between ESCRTs. Sna3 is noteworthy for its ability to enter MVB vesicles without the need for the attachment of Ub to either of its two cytosolic domains (Reggiori & Pelham, 2001). This Ub-independent sorting mechanism depends on the binding of Sna3 to Rsp5, a major E3 Ub ligase for MVB cargoes in yeast (McNatt *et al*, 2007; Oestreich *et al*, 2007a; Watson & Bonifacino, 2007). Rsp5 binds directly to Hse1 of ESCRT-0 (Ren *et al*, 2007) but does not bind to ESCRT-I or ESCRT-II (Bowers *et al*, 2004). This suggests that the subsequent interactions of Sna3 with ESCRT-I and -II are not essential to its sorting into the MVB pathway, although no study has been reported that specifically eliminates a Ub-independent interaction between Sna3 and ESCRT-I and -II. In summary, sequential transfer of MVB cargoes is neither observed nor required for sorting.

Are 'earlier' ESCRTs spatially and temporally further away from forming MVB vesicles? The original intention of the conveyor belt model was to depict the interactions of ESCRTs as a sequence of events that eventually produces MVB vesicles with appropriate cargo proteins. As seen in Fig 2, this temporal order places ESCRT-0 at the furthest position from a forming vesicle, leading to the visual interpretation that it is spatially distant. The subsequent discovery by electron microscopy that MVB vesicle invaginations in mammalian cells do not coincide with the flat clathrin-based membrane coats containing ESCRT-0 (Murk *et al*, 2003) would seem to agree with this spatial assumption. However, an alternative explanation for this observation is that different stages in the vesicle formation process are being represented in the electron microscopy image: ESCRT-0/clathrin coats might potentiate the formation of MVB vesicles but disassemble before the stage of membrane invagination that is observed by electron microscopy (see Supplementary information online, Potentiation of vesicle formation). Other studies indicate ESCRT-0 as the initiator of MVB vesicle formation owing to its roles in recruiting subsequent ESCRTs to endosomes (Katzmann *et al*, 2003; Bache *et al*, 2003). Furthermore, a recent structural study characterized Hrs, the mammalian orthologue of Vps27, as an antiparallel hexameric cylinder with radial symmetry (Pullan *et al*, 2006), which we propose to be more consistent with a role for ESCRT-0 at the centre of the MVB sorting machinery, rather than at the periphery. Indeed, if a similar structure is applied to yeast ESCRT-0, it follows that the association of Sna3 with ESCRT-0-associated Rsp5 would be sufficient for entry of Sna3 into MVB vesicles, positioning Sna3 at the epicentre of MVB vesicle formation. However, we must caution that the low-resolution structure of the Hrs hexamer was obtained in the absence of its binding partner—signal transducing adaptor molecule (STAM)—and the *in vivo* structure of a complete ESCRT-0 complex might be substantially different. Furthermore, the three FYVE (FYVE, 'Fab1, YOTB, Vac1, EEA1') domains at the end of the cylinder are not in a position to bind simultaneously to PI(3)P without the cylinder being significantly embedded in the endosomal membrane bilayer (Pullan *et al*, 2006). Nevertheless, we believe the available data still favour an ESCRT-0 position proximal to forming vesicles, rather than distal.

Apart from ESCRT-0, there is evidence that ESCRT-I lies close to the site of luminal MVB vesicle formation. A significant amount of green fluorescent protein (GFP)-tagged Vps23 is aberrantly packaged into MVB vesicles in cells that lack Mvb12, a previously unknown component of ESCRT-I in yeast (Curtiss *et al*, 2007). Although the

Table 2 | Rules for model construction

1. Should reflect known physical interactions between ESCRT components
2. ESCRTs should assemble in order but need not act strictly linearly
3. Should consider the membrane area required to form a vesicle of appropriate size
4. Should consider spatial requirements on the basis of known ESCRT structures
5. Should selectively incorporate MVB cargoes
6. Deubiquitylation should precede or coincide with vesicle formation
7. ESCRT-II should be able to compensate for the loss of ESCRT-I in yeast
8. ESCRT-III dissociation is not required for MVB vesicle formation
9. Should allow bypass of Ub-dependent cargo-sorting mechanism
ESCRT, endosomal sorting complex required for transport; MVB, multivesicular body; Ub, ubiquitin.

molecular mechanism of this missorting event is not clear, it suggests that ESCRT-I is proximal to the invaginating endosomal membrane. Under the same conditions, GFP-tagged Vps27 is excluded from MVB vesicles (Curtiss *et al*, 2007), which would seem to argue against the idea that ESCRT-0 is at the centre of MVB vesicle formation. A simple explanation for this observation might be that GFP-tagged Vps27 does not function exactly as native Vps27 because expression of the GFP-tagged form exacerbates defects in MVB function that result from mutations in other class E VPS genes (D.P.N. & G.O., unpublished data). Alternatively, Vps27 might not be able to be missorted into MVB vesicles if, as mentioned above, ESCRT-0 dissociates from endosomes before membrane invagination.

Is ESCRT-III proximal to the forming vesicle, and is its dissociation by the ATPase Vps4 the conclusion of vesicle formation? ESCRT-III subunits are highly charged coiled-coil-containing proteins that are predicted to oligomerize into an array or lattice on the endosomal membrane (Babst *et al*, 2002a; Hurley & Emr, 2006; von Schwedler *et al*, 2003). As genetics indicate that ESCRT-III and Vps4 are last in the order of recruitment (Babst *et al*, 2002a), the conveyor belt model suggests that they are last in their order of activities and that dissociation of ESCRT-III by Vps4 is the final event in vesicle formation (Fig 2). Indeed, the fact that all ESCRTs accumulate on endosomes in the absence of Vps4 function but that ESCRT factors are not typically observed in MVB vesicles suggests that Vps4-mediated dissociation must occur before vesicles form (reviewed by Babst, 2005). However, positioning ESCRT-III and Vps4 directly at the site of membrane invagination is not supported by the analysis of Did2, an adaptor protein that couples Vps4 activity to the dissociation of ESCRT-III from endosomes. MVB vesicle formation continues to occur in cells that lack functional Did2, indicating that ESCRT-III dissociation is not required for membrane invagination and budding (Nickerson *et al*, 2006). Despite the persistence of ESCRT-III on endosomal membranes, its subunits cannot be detected inside the luminal vesicles either by immunoelectron microscopy (Nickerson *et al*, 2006) or biochemical methods (M. McNatt, D.P.N. & G.O., unpublished data). This observation suggests that ESCRT-III does not oligomerize on the membrane surface where invagination occurs, but perhaps at the perimeter.

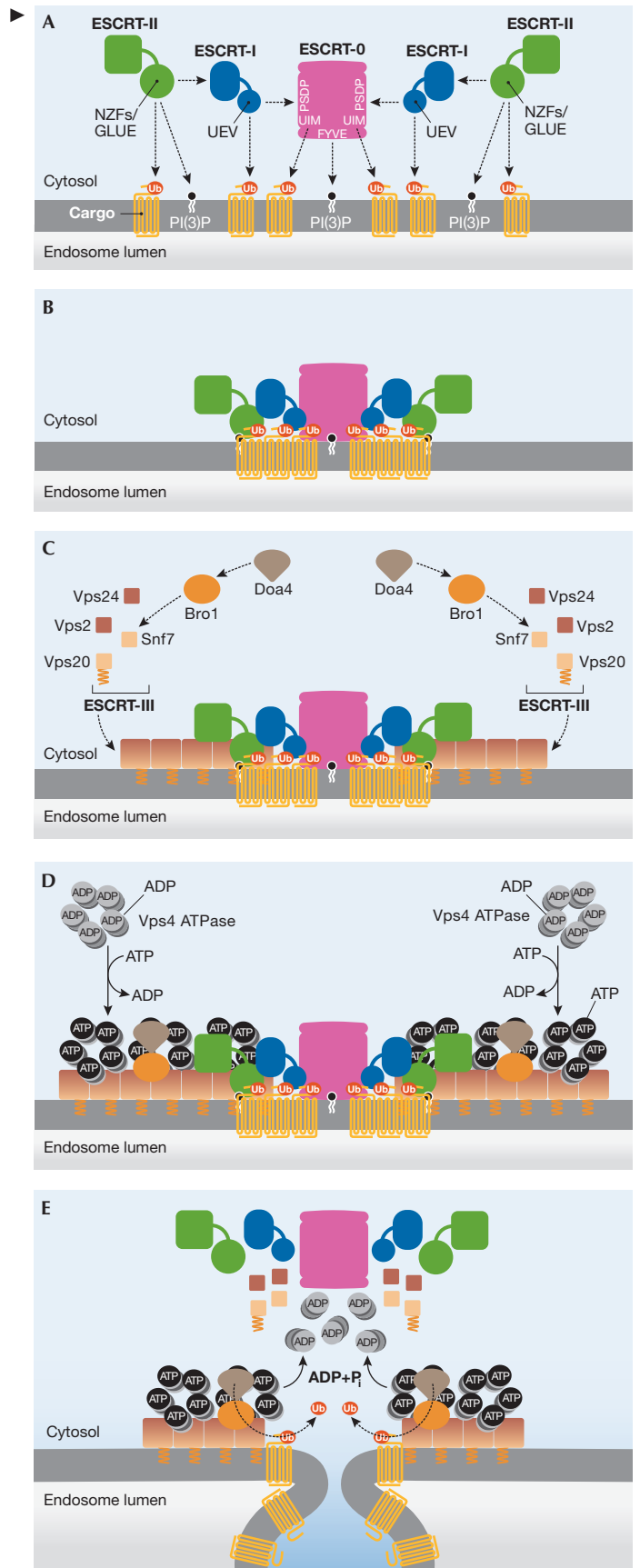
A concentric circle model for ESCRT function

On the basis of the preceding functional studies and logical arguments, we propose a set of rules to govern the construction of an

Fig 3 | Cross-section of concentric circle model of yeast ESCRT function in MVB cargo sorting. (A) Crucial domains of ESCRT-0, -I and -II mediate cargo recognition, lipid binding and complex assembly, resulting in (B) the formation of an ESCRT-0/I/II supercomplex on the endosomal membrane with MVB cargo proteins concentrated beneath. Subunits of ESCRT-III (C) assemble to form a perimeter that recruits both Bro1 and Doa4 and (D) promote assembly of Vps4 dimers into active decamers, although a link between nucleotide exchange in Vps4 and interaction with ESCRT-III is unknown. (E) Dissociation of the ESCRT-0/I/II core precedes vesicle formation, making sequestered MVB cargoes available for Doa4-mediated deubiquitylation before vesicle scission. Vps4 hydrolyses ATP to dissociate ESCRT-III from the membrane after vesicle release. Note that ESCRT-0 is depicted as a barrel having radial symmetry oriented perpendicular to the membrane, although direct evidence for this configuration has yet to be obtained. Bro, BCK1-like resistance to osmotic shock; Doa, degradation of alpha; ESCRT, endosomal sorting complex required for transport; FYVE, 'Fab1, YOTB, Vac1, EEA1'; GLUE, GRAM-like Ub binding in Eap45; MVB, multivesicular body; NZF, Npl4 zinc finger; PI(3)P, phosphatidylinositol 3-phosphate; PSDP, proline serine aspartate proline; Snf, sucrose non-fermenting; Ub, ubiquitin; UEV, Ub E2 variant; UIM, Ub-interacting motif; Vps, vacuolar protein sorting.

alternative model for MVB cargo selection and vesicle formation (see Table 2). Furthermore, we propose that a model of ESCRTs assembling in concentric circles around an ESCRT-0 hub (Figs 3,4) best satisfies these rules. The rationale for ESCRT-0 as the nucleation point for MVB sorting is based on both the data indicating that it has an early function in recruitment of downstream ESCRTs (Katzmann *et al*, 2003; Bache *et al*, 2003) and on the finding that purified Hrs—albeit without its binding partner, STAM—is hexameric and has radial symmetry (Pullan *et al*, 2006). Sequential assembly of ESCRT-I, -II and -III around ESCRT-0 is consistent with known complex interactions (rule 1) and their order of recruitment to the endosome (rule 2). As there is evidence for interactions between disparate ESCRTs, these rings might have 'fuzzy' boundaries; for example, ESCRT-III might have some interaction with ESCRT-I. This model is attractive because it concentrates cargoes within a forming vesicle without the need for transferring them between ESCRT components.

Luminal vesicles in yeast exhibit a range of diameters between approximately 20nm and around 32 nm, with an average of 24 nm (Nickerson *et al*, 2006), which translates to a flat circular membrane area with a diameter of 48 nm. This area provides a membrane template for ESCRT assembly and activity (rule 3). To estimate the space that ESCRTs occupy, we examined the ESCRT domains and cargo that have a 'footprint' on the membrane. This assumption is reasonable as membrane-proximal domains are frequently predicted to connect to their protein complex core by a flexible linker (Hurley & Emr, 2006). Furthermore, the ability to bind to several different partners—including Ub and lipids—is concentrated into these domains, the obvious example being the GLUE domain of Vps36 (Slagsvold *et al*, 2005; Teo *et al*, 2006), suggesting that space at the membrane is limited. The sequential assembly of ESCRT-0, -I and -II each bound to a ubiquitylated MVB cargo yields a membrane footprint that fits comfortably on a 48 nm diameter membrane template (rule 4; also see Supplementary information online, Sizing of ESCRTs). As a working example, if binding occurs between one ubiquitylated cargo and each of the six Vps27 subunits, each of the six Vps23 subunits, and each of the 18 Vps36 subunits depicted in Fig 4, we arrive at a total of 30 ubiquitylated cargoes concentrated by the ESCRT-0/I/II core



when it is functioning at high efficiency in yeast. This cargo load is an underestimate if we also assume that the UIM of Hse1 binds to ubiquitylated cargo, but the stoichiometry of Hse1 interactions with the presumed Vps27 hexamer is unknown.

Assignment of ESCRT-III to the perimeter of the membrane invagination domain provides both a means to sequester these MVB cargoes and exclude non-MVB cargoes (rule 5). The ESCRT-III ring also provides a platform for the activity of Doa4 and therefore allows cargo deubiquitylation (rule 6). Current evidence suggests that ESCRT-0, -I and -II typically dissociate from the endosome before vesicle formation (reviewed by Babst, 2005). This dissociation event would free sequestered cargoes to diffuse laterally in the membrane and bump against the ESCRT-III perimeter to promote deubiquitylation.

The rearrangement of the conveyor belt model into concentric circles suggests a mechanism by which overexpression of ESCRT-II can compensate for the deletion of ESCRT-I in yeast (rule 7). ESCRT-I promotes membrane recruitment of ESCRT-II (Babst *et al.*, 2002b), whereas ESCRT-II interacts with the membrane independently through the binding of its GLUE domain to PI(3)P and can potentially oligomerize (Teo *et al.*, 2006). This suggests that an overabundance of ESCRT-II might form a ring independently of ESCRT-I. Furthermore, because ESCRT-III in this model occupies the perimeter and not the site of membrane invagination, a vesicle might form without the dissociation of ESCRT-III from the cytosolic face (rule 8). As discussed above, the placement of ESCRT-0 at the centre of the invagination domain also positions Sn3 for Ub-independent inclusion in MVB vesicles through its interaction with ESCRT-0-associated Rsp5 (rule 9).

Our concentric circle model presents a different challenge when applied to metazoans because of the difference in scale. Vesicles in HeLa cells, for example, are approximately twice the diameter of those in yeast (Doyette *et al.*, 2005), translating into a membrane surface area with a diameter of 96 nm. This allows for a considerably larger 'core' with more copies of ESCRT-0 and -I at the centre. Accordingly, the Hrs-clathrin network observed in higher eukaryotes (Murk *et al.*, 2003) could reflect an initial need for greater organization across a larger membrane surface area to potentiate vesicle formation, perhaps after clathrin disassembly. Furthermore, we believe this hypothetically larger 'core' in metazoans addresses seemingly contradictory observations that ESCRT-II is essential for vesicle formation in yeast (D.P.N., M. West & G.O., unpublished data) but perhaps not in all metazoan cells (Bowers *et al.*, 2005). It is virtually impossible for the small surface area defined by the ESCRT-0/I core in yeast to form a vesicle, whereas the much larger ESCRT-0/I core described in the metazoan model is sufficiently large to invaginate and release in the absence of ESCRT-II.

Concluding remarks

One possibility arising from the concentric circle model is that contraction of the ESCRT-III ring drives vesicle formation. In this regard, it is intriguing to note the behaviour of the human immunodeficiency virus type 1 (HIV-1) and other enveloped viruses, which usurp the ESCRT machinery to bud virions by a process that is topologically equivalent to the budding of luminal MVB vesicles (reviewed by Morita & Sundquist, 2004). HIV-1 virions arrested in the budding process at the plasma membrane of cells expressing catalytically inactive Vps4^{E235Q} have a neck diameter of more than 100 Å (von Schwedler *et al.*, 2003), which correlates with the predicted 110 Å diameter of a Vps4 multimer (Scott *et al.*, 2005). This suggests that Vps4 might span the virion neck and sever the membrane as it

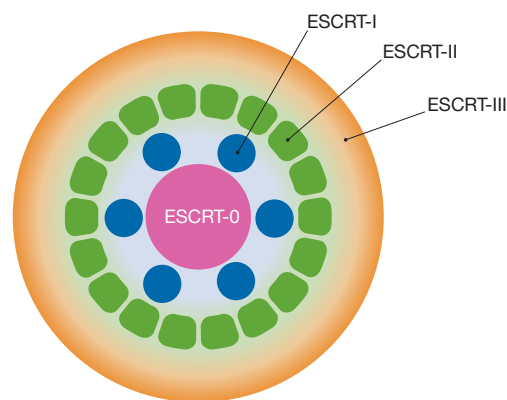


Fig 4 | Top-down view of concentric circle assembly of ESCRT in yeast. With an ESCRT-0 hexamer at the centre, the 48 nm diameter membrane domain accommodates one ring each of ESCRT-I and ESCRT-II, including bound ubiquitylated cargoes. Although the interaction between ESCRT-I and ESCRT-II occurs *in vitro* at 1:1 stoichiometry (Gill *et al.*, 2007), an excess of ESCRT-II is depicted here to account for the potential of ESCRT-II to oligomerize (Teo *et al.*, 2006). ESCRT-III defines the perimeter of the membrane invagination area, oligomerizing outside the ESCRT-0/I/II core. The ESCRT-II/III boundary is depicted as 'fuzzy' owing to the unknown assembly pattern of ESCRT-III and its potential to interact with ESCRT-I. ESCRT, endosomal sorting complex required for transport.

catalyses the dissociation of ESCRT-III. Applying this principle to MVB vesicles, Vps4-mediated contraction of ESCRT-III might be the last step to drive vesicle release.

The concentric circle model makes several predictions that require experimental examination. First, the hexameric structure of a complete ESCRT-0 complex is tentative and requires further investigation in the context of both Vps27/Hrs and Hse1/STAM. Second, our assignment of ESCRT-II to a ring outside of ESCRT-I suggests that ESCRT-II might occupy a larger membrane surface area, resulting in an excess of ESCRT-II to -I on the membrane (Fig 4). Gill *et al.* (2007) showed that ESCRT-I and -II bind directly with a ratio of 1:1 *in vitro*; however, the *in vivo* regulation and arrangement of the two complexes on the endosomal membrane remain unknown, especially in consideration of the ability of ESCRT-II to oligomerize. Third, our assumption that many different ESCRTs simultaneously bind to different ubiquitylated cargoes to position them for inclusion in MVB vesicles predicts that disruption of Ub binding in an individual ESCRT should not block MVB sorting, but instead reduces its efficiency. The development of more rigorous quantitative biochemical assays of MVB cargo sorting should resolve varying degrees of MVB sorting defects. Fourth, we propose that vesicle size is determined by the surface area occupied by the ESCRT-0/I/II assembly surrounded by an ESCRT-III perimeter. Recent studies have shown that a reduction in vesicle size is linked to disrupted Ub regulation at the endosome (McNatt *et al.*, 2007; Richter *et al.*, 2007) and that an increase in size is due to misregulation of ESCRT-III disassembly (Nickerson *et al.*, 2006); however, further work is required to explain the molecular mechanisms of these phenomena. Finally, our proposed radially symmetric, concentric assembly of ESCRTs requires high-resolution imaging of complex assembly on membranes.

The concentric circle model represents the first of a new generation of models of ESCRT assembly and function. We anticipate that the next series of breakthroughs in MVB research will include an explanation of how the interactions between the ESCRT proteins and membrane lipids potentiate vesicle formation (see Supplementary information online, Role of lipids). We also anticipate the discovery of additional MVB sorting mechanisms to supplement the Ub-sorting signal, offering a potential explanation for why some Ub-dependent MVB cargoes display different sorting phenotypes when ESCRT function is attenuated (Bilodeau *et al*, 2002, 2003; Oestreich *et al*, 2007b). The simultaneous presence of three different cargo-recognition complexes within our ESCRT assembly model raises the possibility of several MVB-sorting mechanisms acting at once. As discovered with Sna3, exploring the subtleties of specific cargo selection will lend valuable insight into the larger question of how ESCRTs determine vesicle formation.

Supplementary information is available at *EMBO Reports* online (<http://www.emboreports.org>).

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