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Cell Biology of the ESCRT Machinery

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Abstract

The ESCRT (endosomal sorting complex required for transport) machinery comprises a set of protein complexes that regulate sorting and trafficking into multivesicular bodies *en route* to the lysosome. The physical mechanism responsible for generating luminal vesicles in this pathway is unknown. Here we review recent studies suggesting that components of the ESCRT-III complex drive luminal vesicle formation and consider possible mechanisms for this reaction.

The endosomal system internalizes membrane and protein from the plasma membrane, and then in a topologically distinct process internalizes membrane into itself to create multivesicular bodies (MVBs) that deliver membrane-associated cargo to the lysosome. A set of approximately twenty proteins conserved from yeast to mammals is thought to regulate and drive formation of intraluminal vesicles (ILVs). These include components of four ESCRT (endosomal sorting complexes required for transport) complexes, the AAA+ ATPase VPS4, and several associated proteins. Because the required membrane curvature is opposite to that of coat-mediated transport vesicle formation, it is likely that novel mechanisms are involved. Importantly, the role of the ESCRT machinery extends to topologically similar cellular processes including viral budding, cytokinesis, and possibly autophagy. Interest in the ESCRT machinery has risen over the past few years as it has become clear that perturbations in this pathway contribute to a large number of human diseases ranging from neurodegeneration to cancer. Overall consideration of the ESCRT pathway is provided in several recent reviews [1-5]. Here, we focus on studies that lend insight into the regulation and potential mechanical role of the ESCRT machinery in generating luminal vesicles. We note that there may be additional ESCRT-independent pathways for generating certain types of ILVs in mammalian cells; these are not considered here but are described in earlier excellent papers [6-8].

Overview of the ESCRT machinery

Components of the ESCRT machinery are listed in Table 1. Interfering with the function of any of these proteins in the yeast *Saccharomyces cerevisiae* where they were originally

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discovered leads to missorting of cargo normally destined for the vacuole (the lysosome-equivalent in yeast), causing it to accumulate in abnormal endocytic structures referred to as class E compartments [9]. ESCRT proteins are present throughout eukaryotic evolution, and their role in formation of MVBs is well conserved. Most are components of four ESCRT complexes, ESCRT-0, -I, -II, and -III. Each of these complexes is intrinsically soluble and is recruited transiently to the endosome by interactions with ubiquitinated membrane proteins [10], phosphatidylinositol-3-phosphate (PI(3)P), or other ESCRT complexes [3]. Because it does not end up concentrated inside of luminal vesicles [11,12], the ESCRT machinery must dissociate or be excluded from the vesicle membrane before the vesicle is fully formed and released.

Ubiquitin binding ESCRT machinery:

ESCRT-0, -I, and -II. ESCRT-0, -I, and -II are stable hetero-oligomeric complexes [13-15]. ESCRT-0 is an ~8 nm long heterodimer of HRS and STAM that consists of two short intertwined helical domains joined by an antiparallel coiled-coil [13]. Both HRS and STAM bind ubiquitin and clathrin. HRS additionally has a FYVE zinc-finger domain that binds PI(3)P and PSAP sequence motifs that mediate interaction with Tsg101 in ESCRT-I. ESCRT-I is an 18-25 nm elongated heterotetrameric complex containing one copy each of Tsg101, Vps28, Vps37, and Mvb12 [16,17]. It consists of a headpiece that binds with high affinity to ESCRT-II, an elongated stalk, and a tailpiece that binds ubiquitin and ESCRT-0. ESCRT-II is another heterotetramer made up of one Vps36, one Vps22, and two Vps25 subunits that form a compact Y-shaped complex with a hydrodynamic radius of ~5 nm [18]. Vps36 interacts with PI(3)P, ubiquitin, and ESCRT-I at one end of the complex while Vps25 binds Vps20 to connect the other end to ESCRT-III. If present together on the membrane, these three complexes are expected to cover an area of membrane similar to that needed to form a luminal vesicle [19].

Late acting ESCRT machinery:

ESCRT-III and VPS4. ESCRT-III consists of small, structurally related proteins that assemble into large polymeric structures on the endosomal membrane [20,21]. Yeast has six ESCRT-III proteins, each a 200-250-residue protein with basic N-terminal and acidic C-terminal halves [20]. The ESCRT-III family is expanded to eleven proteins in humans, where they are referred to either as orthologues of their yeast counterparts or as CHMPs (Charged Multivesicular Body Proteins) (see Table 1).

VPS4 is an AAA+ ATPase that releases the ESCRT machinery from the endosomal membrane [14,15,20,22], and as the only energy consuming enzyme directly implicated in the ESCRT pathway has long been considered to be a key factor controlling the creation of MVB vesicles. This thinking is supported by an elegant EM tomography study demonstrating that yeast lacking VPS4 have no ILVs [23]. VPS4 consists of an N-terminal MIT (microtubule interacting and transport) domain connected to a single AAA+ domain [24,25]. It binds to short helical “MIT interacting motifs” (MIM1 and MIM2) in ESCRT-III proteins [26-28]. Like other AAA+ ATPases [29], VPS4 is thought to function as a ring-shaped oligomer that uses alternating movements of central “pore loops” to unfold and thereby disassemble its polymeric ESCRT-III substrates [30,31].

Numerous additional proteins interact with the late ESCRT machinery and affect pathway function. VTA1 and IST1 cooperate with and regulate VPS4 activity [32,33]. Deubiquitinating enzymes, including Doa4 in yeast [34] and AMSH and UBPY in metazoans [35,36], bind to ESCRT-III proteins or the associated Bro1/Alix. While not necessary for internalization *per se*, removing ubiquitin from cargo before delivery into the endosomal lumen helps maintain cellular ubiquitin homeostasis [37].

Sequential action of ESCRT complexes

A framework describing ESCRT function in vacuolar protein sorting grew out of studies that defined an order in which ESCRT components are brought to the membrane and function during MVB biogenesis [20,38]. Structural and biochemical descriptions of interactions between ESCRT complexes followed, supporting the idea that ESCRT complexes could be sequentially recruited into an interconnected network at or near the site of vesicle formation. This model has been described as the “conveyor belt model” and is considered in detail in several excellent reviews [3,19]. In a geometric variant of this termed the “concentric circle model”, sequentially recruited ESCRT components encircle membrane-associated cargo and thereby delineate the content of the incipient ILV [19,39]. Importantly, however, these models do not directly address how the ESCRT machinery helps to physically create a vesicle. It is this question that has recently moved onto center stage.

Beyond cargo sorting: expanding views of ESCRT function

Although the ESCRT machinery was originally identified based on its function in endosomal cargo sorting and ILV formation, it has become clear that it plays an essential role in other cellular processes that share a common membrane topology (Figure 1). First, ESCRT complexes have a well-established role in viral budding. HIV-1, Ebola, and many other enveloped viruses encode structural proteins that recruit components of the ESCRT machinery to facilitate their egress from the cell [40-42]. Impairing VPS4 activity potently inhibits these budding reactions [28,43], supporting an important role for its ESCRT-III substrates in late stages of viral particle release.

Second, the discovery that ESCRT components are required for cell abscission at the end of cytokinesis significantly expands the repertoire of cellular events that utilize this machinery. Mutation or removal of Tsg101, Alix, VPS4, or particular ESCRT-III proteins leads to accumulation of multinucleated cells, and Tsg101 and Alix bind CEP55, a centrosome and midbody protein involved in abscission [44-47]. Alix and Tsg101 compete for a common binding site in CEP55 suggesting that multiple ESCRT-containing complexes may be required for cytokinesis [48]. The finding that archaea of the *Sulfolobus* genus encode proteins related to ESCRT-III and VPS4 that participate in cell division suggests that this might in fact be the original role of the ESCRT machinery [27,49].

Finally, the ESCRT machinery may play a role in generating autophagosomal compartments. The double membrane that sequesters subcellular components – including long-lived proteins, protein aggregates, and organelles – for delivery to the lysosome does not mature normally when ESCRT activity is impaired. Loss of VPS28, VPS25, VPS32 or VPS4 in

Drosophila leads to accumulation of nondegradative autophagosomes [50]. Similarly, loss of Tsg101 or VPS24 in HeLa cells generates ubiquitinated protein aggregates and abnormal autophagic intermediates [51]. In neurons, loss or mutation of ESCRT-0, ESCRT-I, or ESCRT-III components causes the same changes and ultimately neurodegeneration [51,52]. A speculative hypothesis based on topology is that the ESCRTs might play a role in closing the double autophagic membrane [53].

Taken together, the diverse cellular roles now established for the ESCRTs support the emerging hypothesis that this machinery does far more than sort endosomal cargo, promoting physical separation of membrane compartments from each other, including ILV from endosomal membrane during MVB biogenesis, virus from plasma membrane in viral budding, and cell from cell to complete cytokinesis. Evolutionary analyses suggesting that ESCRT-III and VPS4 may comprise the minimal ESCRT machinery [54] together with recent studies of ESCRT-III structure and function focus attention on the role that these late acting components of the ESCRT pathway may play in membrane deformation and possibly vesicle scission.

ESCRT-III function in membrane deformation and scission

A simple model for what we now know about the behavior of individual ESCRT-III proteins is shown in Figure 2. In a “closed” or autoinhibited state each protein is cytoplasmic and probably monomeric, while in an “open” or activated state it is instead on the membrane and part of a polymeric complex [55,56]. VPS4 hydrolyzes ATP to disassemble ESCRT-III polymers, giving directionality to the cycle and returning ESCRT-III proteins to their “closed” and cytoplasmic state. Based on deletion studies [55,56] and the crystal structure of CHMP3 [57], membrane binding and polymer assembly can be attributed to a major N-terminal domain that consists of a 7 nm α -helical hairpin (α 1 and α 2) buttressed on one end by two shorter helices (α 3 and α 4). Interactions with this domain are repressed by C-terminal autoinhibitory sequences that lie in or between helices α 5 and α 6 [55,56,58].

What factor or factors control ESCRT-III cycling? Experimentally, opening can be elicited by deleting C-terminal autoinhibitory sequences [56]. Such constitutively open proteins are potent inhibitors of pathway function. Normally, reversible opening and closing is controlled by specific protein-protein or protein-membrane interactions that affect the autoinhibitory domains. VPS25 in ESCRT-II binds to VPS20's N-terminal domain and probably initiates ESCRT-III assembly [59,60]. Interaction of different ESCRT-III N-terminal domains with each other propagates ESCRT-III assembly [56], while C-terminal sequences recruit VPS4 to disassemble the otherwise stable complexes [26-28,61,62]. Other proteins that bind to C-terminal motifs in individual ESCRT-III proteins, including among others LIP5, Alix, and deubiquitinating enzymes, are also likely to modulate cycling. Not surprisingly, missense or truncation mutations that impair autoinhibitory contacts and/or interaction with regulatory factors have been linked to a variety of diseases including frontotemporal dementia, amyotrophic lateral sclerosis, and familial cataracts (reviewed in [63]).

A turning point in thinking about how ESCRT-III participates in ILV formation came when structural analyses showed that at least some ESCRT-III proteins assemble into membrane-

associated polymers that create or stabilize negative membrane curvature, as needed for events in which the ESCRT machinery participates. Deep-etch electron microscopy (EM) of cells overexpressing human SNF7 proteins showed that each of three related proteins assembles into uniform filaments that curve to form circles and/or spirals on the membrane [64]. The additional finding that these filaments could be induced to generate buds and tubules bending away from the cytoplasm led to the working hypothesis that ESCRT-III might help both to delineate and create ILVs [64,4,19]. EM and crystallography studies of polymers assembled from purified proteins *in vitro* demonstrated that other ESCRT-III proteins also polymerize, and in at least some cases expose a gently concave membrane-binding surface [57,61,62]. These different imaging studies generated much excitement and raised new questions about which type or types of ESCRT-III polymer are relevant to its normal function.

While individual ESCRT-III proteins cycle in and out of polymeric complexes as shown in Figure 2, it has always been clear that each member of the complex has a unique role in the ESCRT pathway. Teis *et al.* recently defined the order with which ESCRT-III proteins have to be recruited to the endosome in yeast ($Vps20 \rightarrow Snf7 \rightarrow Vps24 \rightarrow Vps2$) [21]. Using cross-linker they also captured a transient 450 kDa oligomer that may represent functional ESCRT-III complex [21]. Importantly, Snf7 was the most abundant component of this complex and is also the most abundant ESCRT-III protein in yeast. FRET experiments confirmed that Snf7 subunits are adjacent to each other in the isolated complex. These studies support a model in which Snf7 filaments comprise the bulk of the ESCRT-III complex, and establish that the earlier model of a 1:1:1:1 ratio among ESCRT-III subunits is not likely to be correct [21]. Unique and substoichiometric roles in complex initiation (Vps20), termination (Vps24), and disassembly (Vps2) can be assigned to the other ESCRT-III subunits [21].

Two recent papers elegantly move the study of ESCRT-III *in vitro* [10,65], solidifying working hypotheses about ESCRT-III assembly and function while setting the stage for future mechanistic exploration. In the first, fluorescence spectroscopy is used to follow a series of progressive conformational changes throughout Vps20 and especially Snf7 as they bind to liposomes and to each other on liposomes [10]. EM images of the small liposomes used in this study with bound ESCRT-III show that they invaginate, consistent with the induction of negative curvature by ESCRT-III. In the second study, Hurley and colleagues go a step further and ask how ESCRT-III proteins affect giant unilamellar liposomes [65]. Strikingly, adding purified ESCRT-III proteins to fluorescently labeled liposomes generates a large number of ILVs. This reaction proceeds well with a physiologically defined ratio of Vps20, Snf7, and Vps24; including Vps2 and Vps4 further increases the number of vesicles formed, presumably by recycling the machinery. Key requirements for ILV formation include the use of “pre-activated” Vps20 (lacking autoinhibitory sequences) to bypass the need for upstream initiating factors and of negatively charged phospholipids in the liposomes to recruit and interact with the ESCRT-III proteins.

Taken together, current data supports the idea that ESCRT-III polymers play a direct role in generating luminal vesicles, with Snf7 as their core functional constituent. Much remains to be learned about the underlying mechanism of this unprecedented reaction. Three general

models for how ESCRT-III filaments might be able to create a vesicle have been proposed (Figure 3). All ask how a curved filament on the cytoplasmic surface of the membrane can accomplish this feat without itself ending up concentrated inside of the developing vesicle. In what can be termed the “purse string model” (Figure 3A, [10]), a single ESCRT-III filament surrounds a cargo containing membrane domain. Asymmetric ends of the filament (defined by subunits that initiate or cap the filament) provide a way for VPS4 to remove subunits from one end, shortening the filament while concentrating cargo and inducing the requisite membrane curvature. In a second model (Figure 3B, [65]), the ESCRT-III filament again assembles around an area of membrane containing cargo but now grows as a flat spiral inside of the original circle, constricting cargo into the center to generate a luminal vesicle. Finally, in a third possible model (Figure 3C, [64]), the ESCRT-III filament assembles from the center of a nascent vesicle outwards acting both to bend the membrane and confine cargo as it grows. A key feature of this model is that VPS4 disassembles the tail end of the polymer while the vesicle continues to evolve, effectively creating a moving neck. Much remains to be done to determine which of these or other mechanisms best explains how ESCRT-III functions. Insight into this process will be of fundamental importance and promises to shed light not only on MVB biogenesis but also on viral budding, cytokinesis, and the pathogenesis of a large variety of important diseases.

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Figure 1. Schematic diagram showing multivesicular body biogenesis and topologically related events. The three distinct cellular processes shown - formation of intraluminal vesicles within the MVB, viral budding from the cell surface, and cell abscission at the end of cytokinesis -- share a common membrane topology and depend on at least some aspect of ESCRT pathway function.

Figure 2.

The cycle of ESCRT-III polymer assembly and disassembly. The first four helices of an ESCRT-III protein form a helical bundle responsible for membrane binding and polymerization. These properties are masked by a C-terminal autoinhibitory domain in a "closed" conformation in the cytoplasm. Displacement of this autoinhibitory domain allows ESCRT-III proteins to assume an "open" conformation and assemble into polymers on the membrane. VPS4 hydrolyzes ATP to disassemble these polymers and return the proteins to their "closed" state.

Figure 3.

Models of ESCRT-III driven vesicle formation. (A) "Purse-string" model based on [10]. A single ESCRT-III filament with asymmetric ends is used to delineate and later constrict the neck of an evolving vesicle. VPS4 is proposed to disassemble the filament from one end to constrict the string. (B) "Spiral constriction" model based on [65]. A growing ESCRT-III spiral surrounds and eventually constricts a cargo containing membrane domain, forcing cargo at the center into an evolving vesicle. (C) "Moving neck" model based on [64]. ESCRT-III filaments start at the center of a developing vesicle, bending the membrane as they grow. Disassembly of the filament tail allows the spiral to remain in only the neck of the evolving vesicle.

Table 1

ESCRT pathway components. Names and synonyms for ESCRT pathway proteins in the yeast *Saccharomyces cerevisiae* and in metazoans. The most prevalent name for the human proteins in the current literature is listed as the primary name.

Complex	Yeast proteins	Yeast protein Synonyms	Metazoan proteins	Metazoan protein Synonyms
ESCRT-0	Vps27		Hrs	VPS27
	Hse1		STAM1, 2	HSE1, 2
ESCRT-I	Vps23		Tsg101	VPS23
	Vps28		VPS28	
	Vps37		VPS37A, B, C, D	
	Mvb12		MVB12A, B	
ESCRT-II	Vps22		VPS22	EAP30
	Vps25		VPS25	EAP20
	Vps36		VPS36	EAP45
ESCRT-III	Vps2		CHMP2A, B	VPS2-1, 2
	Vps24		CHMP3	VPS24
	Vps20		CHMP6	VPS20
	Snf7	Vps32	CHMP4A, B, C	SNF7-1, 2, 3
	Vps60	Mos1	CHMP5	VPS60
	Did2	Vps46	CHMP1A, B	DID2-1, 2
	-	-	CHMP7	
Others	Vps4		VPS4A, B	SKD1 (VPS4B)
	Vta1		LIP5	VTA1
	Ist1		IST1	
	Bro1	Vps31	ALIX	AIP1