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ESCRT-dependent cargo sorting at multivesicular endosomes

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Abstract

The endosomal sorting complex required for transport (ESCRT) machinery is composed of five multi-subunit protein complexes, which act cooperatively at specialized endosomes to facilitate the movement of specific cargoes from the limiting membrane into vesicles that bud into the endosome lumen. Over the past decade, numerous proteins, lipids, and RNAs have been shown to be incorporated into intralumenal vesicles (ILVs), but the mechanisms by which these unique cargoes are captured are only now becoming better understood. Here, we discuss the potential roles that the ESCRT machinery plays during cargo sorting at multivesicular endosomes (MVEs).

Keywords

ESCRT machinery; multivesicular endosome; cargo sorting; intralumenal vesicle; ubiquitin

1. Introduction

Eukaryotic cells have evolved elaborate mechanisms to sense environmental cues, rapidly initiate responses mediated by cell surface transmembrane receptors, and ultimately downregulate signaling via receptor sequestration within endosomal compartments. This pathway was originally described several decades ago in elegant electron microscopy-based studies using labeled epidermal growth factor (EGF)[1–4], which is internalized rapidly into cells and deposited into multivesicular endosomes (MVEs; also referred to as multivesicular bodies/MVBs). Identification of MVEs at the ultrastructural level is made relatively simple by their characteristic morphology: membrane-bound organelles that harbor small intralumenal vesicles (ILVs). To generate these unique membrane compartments, eukaryotes use a set of protein complexes collectively known as the Endosomal Sorting Complex Required for Transport (ESCRT) machinery, which were defined originally in a series of genetic screens using yeast[5–10]. In the absence of ESCRT function, transmembrane cargoes are unable to efficiently reach the lumen of the vacuole/lysosome, resulting in the formation of an aberrant prevacuolar organelle in yeast termed the ‘class E compartment’. Since their initial discovery, the field has begun to reach consensus regarding the roles of the ESCRT

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complexes at the endosome limiting membrane, which include cargo selection, membrane deformation to generate nascent, inward-budded vesicles, cargo sequestration within these newly formed vesicles, and narrowing of the vesicle bud neck to ultimately facilitate release of ILVs into the lumen of the endosome.

During the characterization of these processes, several model cargoes were identified as substrates of the ESCRT machinery. Perhaps most famously, post-translational modification of the EGF receptor by ubiquitin was shown to play an integral role in its endosomal sorting and degradation[11–14]. Together with other seminal discoveries linking ubiquitin to the ESCRT machinery at MVEs, the trajectory for the field was set, and a unifying mechanism that supports the entry of numerous, unrelated cargoes into the endosome lumen was defined[8,15–18]. More recently, additional cargoes that do not rely on ubiquitin modification have also been shown to enter ILVs, including proteins (both soluble and transmembrane), specialized lipids, and nucleic acids, suggesting that multiple modes of cargo recognition exist[19–24]. Furthermore, the fate of MVEs has been expanded beyond lysosomal fusion and ILV degradation. In particular, some MVEs are specialized to facilitate the dissemination of cargoes to the extracellular environment, fusing with the plasma membrane to release their ILVs (exosomes)[25].

The spatial distribution of MVEs can vary widely between cell types, and probes specific to the organelle have been challenging to identify, as they regularly undergo fusion with lysosomes and other membrane compartments. Similarly, the size and content of MVEs are not uniform and depend on cell cycle stage, nutrient availability, and other environmental conditions. Components of the ESCRT machinery may represent the best markers available, given their intimate role in MVE biogenesis. In general, mammalian MVEs tend to accumulate near the center of cells at steady state[26], in a manner dependent on dynein-mediated transport[27], which positions them well to receive biosynthetic cargoes from the perinuclear trans-Golgi network. However, a large number of cargoes also originate at the cell surface, entering the endosomal system via clathrin-dependent and clathrin-independent endocytosis, phagocytosis, and micropinocytosis[28–31]. Based on live cell total internal reflection microscopy (TIRFM), components of the ESCRT machinery have been observed at clathrin-coated structures at or near the plasma membrane, suggesting that cargoes are sorted rapidly upon cellular internalization[32]. Thus, MVE formation likely initiates at multiple subcellular locations to sequester cargoes destined for degradation or exocytosis. Perhaps not surprisingly, growth factor stimulation augments the rate of MVE formation, enabling a rapid response to limit cell signaling that is initiated upon receptor binding[33–35].

Based on genetic and biochemical evidence, more than two dozen ESCRT proteins have been implicated in the deposition of cargoes into ILVs[36]. In many cases, structural and functional evidence points to mechanisms by which these factors participate in cargo sorting. For example, early acting ESCRT complexes (ESCRT-0, ESCRT-I, and ESCRT-II) harbor ubiquitin-binding domains, which play a key role in cargo selection[37]. However, it remains unclear how (and to what extent) substrates of the pathway, but not core components of the ESCRT machinery, become selectively enriched within ILVs. Here, we will focus on this issue and discuss potential models to explain how ESCRT-mediated cargo sorting is efficiently achieved.

2. Ubiquitin-mediated cargo recognition and sorting by the ESCRT machinery

Post-translational modification of integral membrane proteins by ubiquitin serves as a key sorting signal for endocytosis and targeting to MVEs. In particular, members of the Cbl family of E3 ubiquitin ligases have been tied to the internalization and ESCRT-dependent degradation of dozens of activated cell surface receptors[11,38–40]. Although the addition of a single ubiquitin moiety has been shown to be sufficient to enable ESCRT-mediated protein sorting at MVEs[41], many cargoes including EGF receptor undergo polyubiquitin modification (predominantly via K63-linkages), which may enhance their rate of sequestration within ILVs[18,42–44]. Several components of the ESCRT machinery contain ubiquitin binding domains (UBDs), which act as receptors for ubiquitin-modified cargoes (Figure 1). In general, the UBDs found on ESCRT subunits exhibit only modest affinity (K_d of ~70–510 μM) for cargoes[45], but the avidity of these interactions may be sufficient to play a significant role in cargo capture and retention, especially when considering that integral membrane proteins are constrained to two dimensional movement within a bilayer[46]. The two subunits of ESCRT-0 (Hrs and STAM) contain two UBDs each. The double ubiquitin interacting motif (DUIM) of Hrs can bind simultaneously to two ubiquitin molecules[47], each with an affinity of ~120 μM[48], and both the UIM and Vps27/Hrs/STAM (VHS) domains of STAM associate with ubiquitin[49], albeit with weaker affinities[48,50]. Importantly, upon association with membranes, ESCRT-0 exhibits the ability to generate larger complexes, further increasing the number of UBDs presented[48,51]. Both fluorescence and immunogold electron microscopy-based studies in yeast and animal cells support the idea that ESCRT-0 self-associates at endosomal membranes, creating subdomains that facilitate the retention of ubiquitin-modified cargoes through multiple low-affinity associations[52–54]. Additionally, ESCRT-0 subdomains may be further stabilized by flat clathrin lattices that are recruited by the carboxyl-terminal clathrin binding motif of Hrs[18,53,55]. However, a universal requirement for clathrin assembly at MVEs during cargo sorting remains to be demonstrated in all cell types. In the absence of clathrin heavy chain, biosynthetic transport of lysosomal hydrolases into MVEs in yeast proceeds normally[56–58]. Additionally, subdomains of ESCRT-0 are capable of assembling stably in the absence of flat clathrin lattices in vitro[59]. One possibility is that clathrin assembly at MVEs increases the efficiency of cargo retention, which may be required in cases where cargo influx becomes elevated in response to developmental and extracellular cues.

In contrast to ESCRT-0, no evidence exists to suggest that the heterotetrameric ESCRT-I complex multimerizes on membranes[51]. Nevertheless, based on structural and functional studies, at least two of its components can associate directly with ubiquitin. The core subunit Tsg101 possesses an amino-terminal, catalytically inactive ubiquitin E2 variant (UEV) domain with weak affinity for ubiquitin (K_d of ~510 μM)[60]. Additionally, a subpopulation of ESCRT-I complexes contain the UBAP1 subunit, which harbors a solenoid of overlapping ubiquitin associated motifs (SOUBA domain) that binds mono- and di-ubiquitin with an affinity of ~70 μM[61]. Inhibition of UBAP1 leads to cargo-specific trafficking defects, indicating that it (and by extension, ESCRT-I) plays a role in ubiquitin-dependent sorting[62]. The ESCRT-II complex contains a single subunit (Vps36) capable of binding to ubiquitin via

a GRAM-like ubiquitin-binding in Eap45 (GLUE) domain[63]. Similar to other UBDs found in ESCRT subunits, the affinity of the GLUE domain for ubiquitin is modest (K_d of ~330 μM)[63]. However, ESCRT-II has been shown to multimerize on membranes in a cholesterol dependent manner, which would elevate its avidity for ubiquitin-modified cargoes[64]. Additionally, recent evidence in yeast highlighted a mutant form of ESCRT-II that is capable of partially suppressing phenotypes associated with the loss of ESCRT-0 or ESCRT-I, implying a direct role for ESCRT-II in cargo sorting at MVEs[65].

Recruitment of ESCRT-III to endosomes is mediated largely by ESCRT-II, via a direct interaction between the ESCRT-II subunit Vps25 and the ESCRT-III subunit Vps20[66]. However, additional factors have also been identified, which can bridge earlier acting ESCRT complexes with ESCRT-III. In particular, ALIX has been shown to interact with both ESCRT-I (via Tsg101) and Vps32 isoforms[67–71]. Moreover, ALIX harbors a UBD (UBAN-like), which may contribute to cargo sorting into ILVs[72–74]. Although defects in cargo trafficking are relatively mild in the absence of ALIX function, they become more pronounced when other components of the ESCRT machinery are compromised[75–77], suggesting that ALIX contributes to the overall efficiency of cargo delivery into MVEs.

The ESCRT-III and Vps4 complexes lack the presence of UBDs and their contribution to ubiquitin-dependent cargo sorting has been challenging to reconcile. One compelling hypothesis is that ESCRT-III spiral filaments may encircle cargoes to physically restrict their diffusion on MVEs[78,79], although direct evidence for such a model is currently lacking. The ability of ESCRT-III to corral cargoes may be particularly important considering that substrates of the ESCRT pathway are typically subject to deubiquitination prior to internalization into ILVs. In yeast, the ubiquitin peptidase Doa4 has been demonstrated to associate with the ESCRT-associated protein Bro1 (an orthologue of mammalian ALIX), Vps20 and Vps32[80–83]. In particular, Vps20 binding to Doa4 appears to inhibit its deubiquitinase (DUB) activity, thereby delaying removal of ubiquitin from cargoes, perhaps until ESCRT-III spiral arrays can assemble properly[84]. In mammalian cells, several ESCRT-III subunits (CHMP1, CHMP2, CHMP3, and Ist1) associate with deubiquitinating enzymes, including AMSH, which specifically cleaves K63-linked ubiquitin chains[85–91]. However, unlike Doa4, the precise role of AMSH during cargo sorting remains to be clearly defined.

Despite tremendous advances in our understanding of ubiquitin-dependent cargo sorting at MVEs, the key question of how components of the ESCRT machinery function cooperatively to ensure that specific cargoes are properly deposited into forming ILVs remains unanswered. Although ESCRT-0 can bind to ESCRT-I (via a direct association between Hrs and Tsg101), and ESCRT-I can associate with ESCRT-II (via a direct association between the ESCRT-I subunit Vps28 and Vps36), localization studies have failed to demonstrate extensive co-localization between the complexes on endosomes[10,92–94]. Additionally, there exists little direct evidence to support the idea that these complexes co-assemble to form a higher order assembly in vivo. Instead, each complex may participate independently in cargo binding and due to their low affinities for ubiquitin, rapidly transfer cargoes to one another via transient associations. Based on live cell imaging studies, the majority of ESCRT-0 is found on endosomes, while ESCRT-I and ESCRT-II exhibit a more diffuse cytoplasmic distribution[95–98], consistent with biochemical data showing that ESCRT-

I and ESCRT-II are soluble[99]. Thus, ESCRT-0 has been suspected to play the most significant role in ubiquitin-dependent cargo clustering at endosomes. Consistent with this idea, an auto-activated, mutant isoform of the yeast ESCRT-III subunit Vps32 can largely bypass the requirements for ESCRT-I and ESCRT-II function in cargo sorting, but not ESCRT-0[100]. Additionally, in vitro studies suggest that ESCRT-0, but not ESCRT-I or ESCRT-II, is capable of stably associating with ubiquitin-modified substrates on supported lipid bilayers[51]. Together, the current evidence suggests a model in which ESCRT-0 functions as the major sorting receptor for ubiquitin-modified substrates at MVEs, with ESCRT-I and ESCRT-II augmenting its actions to create subdomains enriched with select cargoes, and linking the early acting ESCRT machinery to the downstream ESCRT-III complex.

3. Ubiquitin-independent cargo sorting

In recent years, there have been numerous studies indicating that integral membrane proteins lacking ubiquitin modification can still be internalized into MVEs in an ESCRT-dependent fashion. The G protein-coupled protease-activated receptor-1 (PAR1) and the purinergic receptor $P2Y_1$ both contain a YPX_3L motif, to which the ESCRT-associated protein ALIX binds[19,20]. Similarly, the interleukin-2 receptor β (IL-2Rβ) interacts directly with Hrs in a manner that does not require ubiquitin[22]. The mechanisms by which Hrs and ALIX translocate substrates into ILVs is unclear, but their internalization continues to require the downstream ESCRT machinery (ESCRT-III and the Vps4 complex)[19,20].

There have also been reports of soluble cargoes entering ILVs for degradation. In one case, selectivity of cargoes is achieved by the chaperone hsc70, which associates with the limiting membrane of MVEs through a polybasic motif that interacts with phosphatidylserine[101]. Although unfolding of substrates is unnecessary for deposition into ILVs, both early (ESCRT-I) and late acting (ESCRT-III and Vps4) components of the ESCRT machinery are required. Additionally, association of soluble cargoes with proteins heavily modified by ubiquitin, such as members of the Cos family of tetraspanin-like proteins, can lead to their internalization within ILVs in an ESCRT-dependent manner[102].

Beyond the sorting of proteinaceous cargoes, several studies have demonstrated that specific lipid species are preferentially sorted into MVEs. In particular, the phospholipids lysobisphosphatidic acid (LBPA) and phosphatidylinositol 3-phosphate (PI3P) are abundant on ILVs[23,103]. The ESCRT associated protein ALIX binds directly to LBPA[21,104], and depletion studies suggest that ALIX promotes LBPA enrichment specifically at sites of ILV formation[21]. In vitro, LBPA stimulates membrane deformation on synthetic liposomes[21], and it may behave similarly in cells to drive or stabilize the high curvature characteristic of ILVs in mammals (~50–60 nm in diameter). PI3P similarly associates with components of the ESCRT machinery, including ESCRT-0 (via the Hrs FYVE domain)[105] and ESCRT-II (via the Vps36 GLUE domain)[106]. In particular, Hrs associates with PI3P with strong affinity[107], and the lipid likely facilitates the formation of ESCRT-0 subdomains on MVEs. Additionally, other ESCRT components, including several ESCRT-III subunits, preferentially interact with acidic phospholipid headgroups like that found on PI3P[108]. However, given that PI3P becomes enriched in ILVs, it remains unclear how components of

the ESCRT machinery ultimately escape the degradative fate of a major interacting lipid partner. In fact, contrary to studies suggesting that ESCRT components are usually recycled[10], proteomic studies of exosomes have consistently identified several components of the ESCRT machinery[109–111], indicating that recycling of ESCRT subunits may not be as robust as once believed.

In recent years, exosomes have garnered wide interest, based on their involvement in cell-to-cell communication and their roles in immune system regulation, cancer biology, and nervous system development[112]. The analysis of purified exosomes has revealed the presence of not only proteins and lipids, but several classes of RNAs, including mRNAs and miRNAs[113]. The mechanisms by which specific RNAs accumulate in ILVs remain unclear. ESCRT-II was shown previously to associate with the 3′ UTR of bicoid mRNA via the Vps36 GLUE domain to direct its localization in Drosophila eggs[114]. However, since neither ESCRT-I nor ESCRT-III inhibition affected bicoid mRNA distribution, it was believed that the role of ESCRT-II in this process was independent of an endosomal sorting function[114]. More recent work suggested that the RNA binding protein YBX1 acts as a molecular chaperone to package a subclass of miRNAs into ILVs[115]. Surprisingly, YBX1 is not membrane bound. Instead, it is largely associated with cytoplasmic RNA granules, which must intersect with MVEs to deliver specific miRNAs into ILVs[116]. It is possible that YBX1 is ubiquitin modified and/or capable of associating directly with components of the ESCRT machinery or other ubiquitin-modified integral membrane proteins, but additional studies are required to gain a mechanistic understanding of its function.

4. Cargo transfer into ILVs

With several lines of evidence supporting the idea that the early acting ESCRT complexes promote initial cargo sorting and clustering on MVEs and the late acting components restrict cargo diffusion prior to their incorporation into ILVs, a major unresolved issue is the mechanism by which cargoes are efficiently transferred between these ESCRT complexes. Two main models have emerged to explain this phenomenon. In the concentric circle model, cargoes are initially retained within a subdomain established by a combination of ESCRT-0, ESCRT-I and ESCRT-II[78]. Subsequent nucleation of ESCRT-III complexes by ESCRT-II results in the formation of a flat spiral polymer that surrounds cargoes, which rapidly adopts an energetically unfavorable conformation, leading to mechanical tension on the membrane[117,118]. Ultimately, the polymer is forced to convert into a three-dimensional helix, deforming the membrane to generate a nascent vesicle, which harbors the cargoes found at its core. To prevent internalization of the early acting ESCRT machinery in this model, each complex must be released from the membrane prior to bud formation (Figure 2). Currently, there exist no data to suggest how displacement of ESCRT-0, ESCRT-I, and ESCRT-II from the limiting MVE membrane is achieved. Nonetheless, there are several testable predictions based on this model. First, the early acting ESCRT machinery should not form stable, long-lived subdomains, but instead generate transient patches on MVEs that turnover with each cycle of ILV formation. Second, the early acting ESCRT complexes should at least transiently co-localize with late acting ESCRT components on MVEs. Finally, ESCRT-III spiral filaments should grow in an outward manner to generate the necessary compression forces on membranes. Although the live cell imaging studies

necessary to address the first two predictions have yet to be reported, multiple groups have examined the third in vitro. In both cases, ESCRT-III polymers, consisting largely of Vps32 subunits, were found to exhibit a growing diameter, from ~25 nm at their center to several micrometers at their periphery, consistent with outward polymerization[117–119]. These data are supported by other studies indicating that Vps32 polymers exhibit a preference for a high radius of curvature[120]. However, it remains unclear how other ESCRT-III subunits may influence the initial orientation of polymer assembly. If the nucleation process led to the formation of inward spiraling filaments, the ESCRT-III complex could assemble adjacent to a subdomain composed of the early acting ESCRT complexes (Figure 2). In this scenario, it is easy to recognize how ESCRT components would avoid engulfment, but it becomes more challenging to comprehend how cargoes are transferred between the early acting ESCRT machinery and a neighboring ESCRT-III polymer. One possibility is that cargoes are only transiently retained by ESCRT-0, ESCRT-I and ESCRT-II, consistent with their modest affinity for ubiquitin sorting signals. This would enable cargoes to diffuse into juxtaposed subdomains that become encircled by ESCRT-III spiral filaments, targeting them into ILVs. High resolution imaging of native ESCRT complexes will be necessary to resolve these conflicting models.

5. Concluding perspectives

In the majority of intracellular protein sorting reactions, receptors play an integral function in concentrating or retaining specific cargoes within budding transport carriers. For example, during COPII-mediated transport, members of the Sec24 or Tango1 cargo receptor families play intimate roles in directing specific substrates out of the endoplasmic reticulum[121,122]. However, the ESCRT machinery acts at the neck of nascent vesicles. While potentially sufficient to restrict cargo escape, this distribution diminishes the ability to prevent the non-specific inclusion of bulk cytoplasmic proteins and nucleic acids within ILVs. Nonetheless, the contents of ILVs appear to be highly selective, suggesting the existence of mechanisms to enrich for specific cargoes. Notably, other regions of the endosomal membrane are also active in cargo sorting. Specifically, retromer activity and other recycling pathways simultaneously regulate the content of endosomal membranes[123]. Only through the cooperative actions of multiple cargo sorting systems is ILV content appropriately maintained. In the future, continuing improvements to live cell imaging technologies and CRISPR-mediated genome editing approaches to label endogenous ESCRT components will enable us to gain a better understanding of the dynamic actions of the ESCRT machinery at endosomes, which should address many of the outstanding questions that remain in this field.

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Abbreviations

DUB deubiquitinating enzyme

DUIM	double ubiquitin-interacting motif
EGF	epidermal growth factor
ESCRT	endosomal sorting complex required for transport
GLUE	GRAM-like ubiquitin-binding in Eap45
ILV	intralumenal vesicle
LBPA	lysobisphosphatidic acid
MVB	multivesicular body
MVE	multivesicular endosome
PI3P	phosphatidylinositol 3-phosphate
SOUBA	solenoid of overlapping ubiquitin associated motifs
TIRFM	total internal reflection fluorescence microscopy
UBD	ubiquitin binding domain
UEV	ubiquitin E2 variant
UIM	ubiquitin-interacting motif
VHS	‘Vps27, HRS, STAM’
Vps	vacuolar protein sorting

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ESCRT Complex	ESCRT Subunit	Ubiquitin binding domain
ESCRT-0	Hrs STAM	DUIM UIM, VHS
ESCRT-I	Tsg101 UBAP1	UEV SOUBA
ESCRT-II	Vps36	GLUE

Figure 1. The early acting ESCRT machinery harbors multiple ubiquitin binding domains

Several components of ESCRT-0, ESCRT-I and ESCRT-II contain domains capable of low affinity association with ubiquitin.

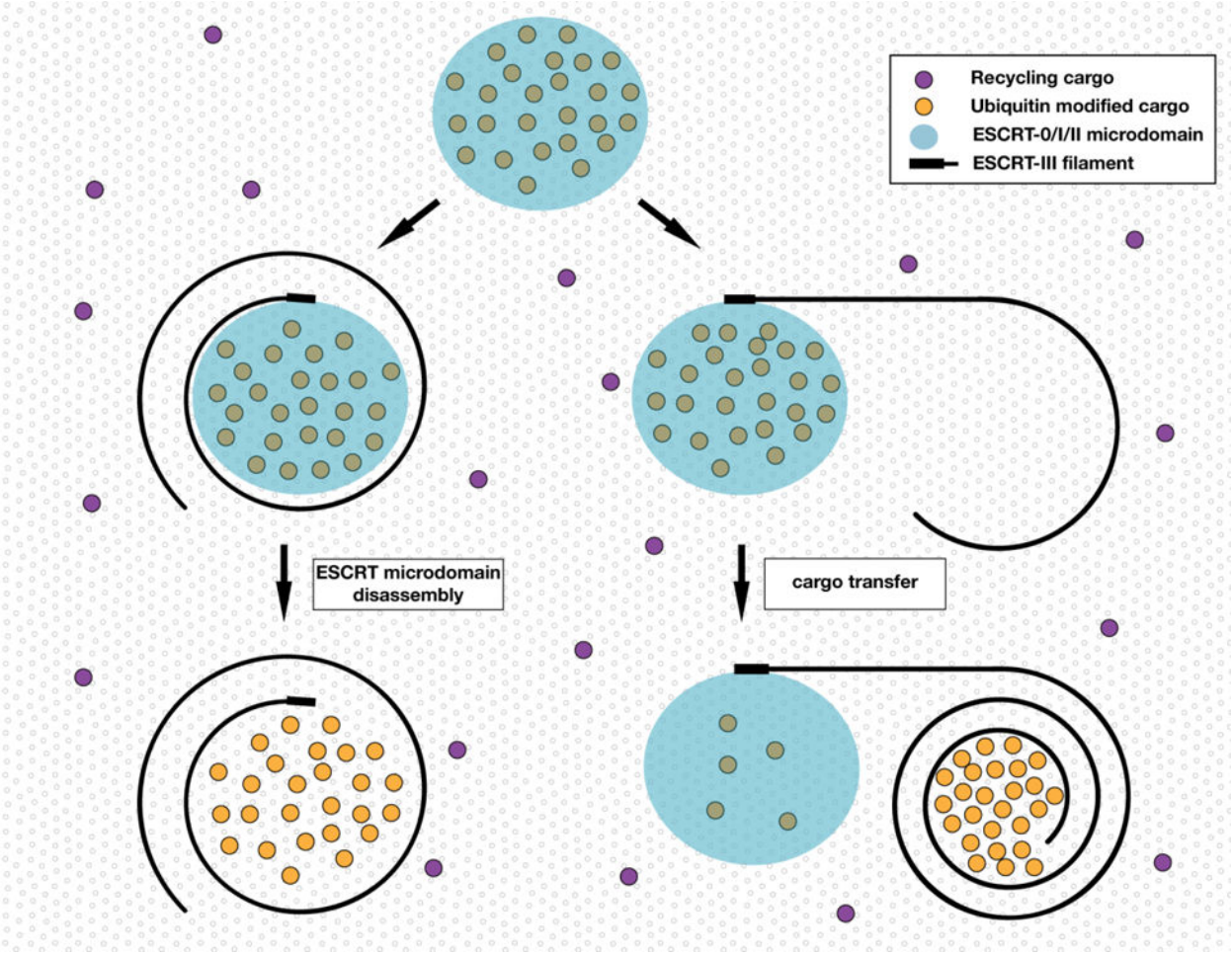

Figure 2. Two speculative models to describe pathways for ESCRT-III spiral filament assembly

In one model (left), a subdomain formed by the early acting ESCRT machinery nucleates ESCRT-III filaments that spiral outward to surround cargoes destined for incorporation into ILVs. In this model, ESCRT-0, ESCRT-I and ESCRT-II must dissociate from the endosomal membrane to avoid engulfment into ILVs. An alternative model (right) suggests that the early acting ESCRT machinery forms a subdomain adjacent to ESCRT-III filaments, which spiral inwards toward a preferred radius of curvature. In this case, cargoes must be transferred between the early and late acting ESCRT complexes.