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Review

The ESCRT Pathway

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Multivesicular bodies (MVBs) deliver cargo destined for degradation to the vacuole or lysosome. The ESCRT (endosomal sorting complex required for transport) pathway is a key mediator of MVB biogenesis, but it also plays critical roles in retroviral budding and cytokinetic abscission. Despite these diverse roles, the ESCRT pathway can be simply seen as a cargo-recognition and membrane-sculpting machine viewable from three distinct perspectives: (1) the ESCRT proteins themselves, (2) the cargo they sort, and (3) the membrane they deform. Here, we review ESCRT function from these perspectives and discuss how ESCRTs may drive vesicle budding.

Introduction

The formation of multivesicular bodies (MVBs) is a key stage in the delivery of cargo destined for degradation in the yeast vacuole or mammalian lysosome. MVBs were discovered in the 1950s by Keith Porter and George Palade when they observed "two large vesicles with smaller vesicles inside" in electron micrographs (Palade, 1955; Sotelo and Porter, 1959). In the late 1970s, Stanley Cohen and colleagues observed the sorting of epidermal growth factor receptor (EGFR) into the intraluminal vesicles (ILVs) of endosomes, similar to the structures previously reported by Porter and Palade (Haigler et al., 1979). Despite these early observations, the machinery responsible for MVB biogenesis was identified only recently.

In 2001, the endosomal sorting complex required for transport-I (ESCRT-I) complex was characterized and shown to engage ubiquitinated cargo at the endosome and mediate its sorting into MVBs (Katzmann et al., 2001). A year later, two back-to-back papers in *Developmental Cell* identified the ESCRT-II and ESCRT-III complexes as critical players in the delivery of ubiquitinated cargo to the yeast vacuole (Babst et al., 2002a, 2002b). These studies were preceded by the identification of the AAA ATPase Vps4 (part of a fifth ESCRT complex) in 1997, which is necessary for delivery of cargo to the vacuole (Babst et al., 1997). Together, these papers set the framework establishing the ESCRT proteins as cargo sequestering and sorting machinery that can deform the endosomal-limiting membrane inward to generate MVBs.

These studies were also significant in that they organized and assigned individual functions to the earlier identified "class E" vacuolar protein sorting (*vps*) genes that, when knocked out in yeast, fail to deliver cargo to the vacuole and exhibit aberrant endosome morphology (Raymond et al., 1992). They also initiated work demonstrating that the ESCRTs comprise a pathway of five distinct complexes (ESCRTs -0, -I, -II, and -III, and Vps4) that recognize and sort ubiquitinated cargo through an exquisite division of labor.

Today, however, the ESCRT field includes more than studies of endosomal trafficking. Recent work has elucidated roles for the ESCRTs in numerous biological processes. Beyond MVB formation, ESCRT proteins are well established to function in eukaryotic cell abscission and viral budding (Figure 1), as well as exosome secretion, and autophagy (Carlton et al., 2008; Filimonenko et al., 2007; Lee et al., 2007; Rusten et al., 2007). Although not the focus of this review, ESCRT dysfunction is associated with numerous diseases, including cancer, neurodegeneration, Huntington's, and Parkinson's diseases (for review, see Saksena and Emr, 2009).This review cannot adequately cover all the roles ESCRTs play in the cell. Rather, the purpose here is to emphasize the key findings that define ESCRT function. These discoveries highlight "unifying principles" of the ESCRTs, which are shared in some capacity between processes. At the most basic level, these principles define the ESCRT pathway as a cargo-recognition and membrane-deformation machine that can be viewed from three perspectives: (1) the ESCRTs themselves, (2) the cargo they sort, and (3) the lipid membrane they deform (Figure 2). We have structured this review to focus on each of these three perspectives. At the same time, there is an apparent divergence in ESCRT function regarding these different processes, as well as differences in ESCRT complexes between yeast and metazoans. Divergence is most apparent in the differential requirements for some ESCRT proteins over others in certain biological contexts. Careful analysis of these differences provides insight into the mechanisms of ESCRT function. Finally, we will discuss the leading models explaining how ESCRT-III, the least understood of the ESCRT complexes, achieves vesicle formation. Thus, this review is meant to both reflect on ESCRT discoveries as well as discuss key mysteries remaining in the field. For more in-depth discussion, the reader should refer to several reviews that focus on ESCRT structure, ESCRT roles in diseases, the ubiquitin cycle, and vesicle budding (Hurley et al., 2010; Raiborg and Stenmark, 2009; Saksena and Emr, 2009; Williams and Urbé, 2007).

The ESCRT Complexes

ESCRTs were first defined as a ubiquitin-dependent protein-sorting pathway in yeast. Earlier studies in the 1980s and 1990s in the Emr and Stevens laboratories had identified numerous genes in *Saccharomyces cerevisiae* yeast that, when lost, displayed *vps* defects (Banta et al., 1988; Robinson et al., 1988; Rothman et al., 1989; Rothman and Stevens,

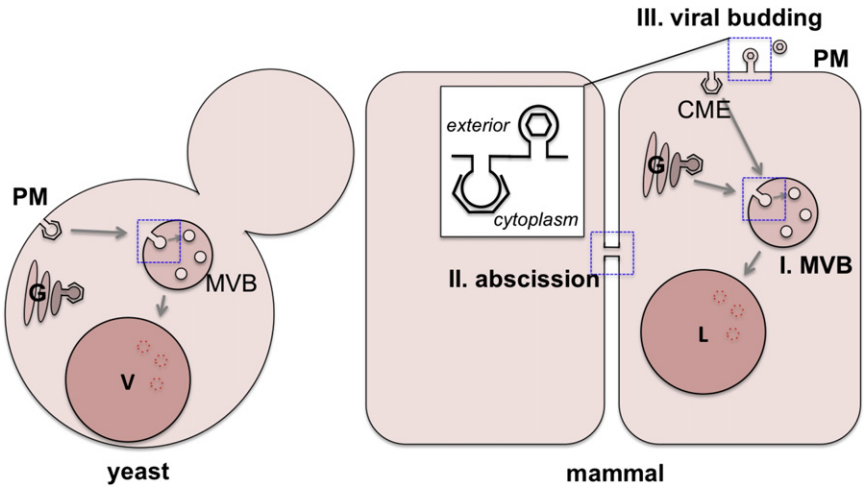


Figure 1. ESCRT-Mediated Processes in Yeast and Mammals
The left view shows the ESCRT pathway in yeast delivering cargo from the plasma membrane (PM) or *trans*-Golgi (G) to the vacuole (V) via MVBs. The right view illustrates mammalian cells utilizing ESCRTs in at least three distinct ways. MVB formation (I) delivers cargo to the lysosome (L). ESCRTs are also used in the abscission event of cell cytokinesis (II). Some viruses also use ESCRT proteins to bud from the plasma membrane (III). In the inset, this budding is topologically inverted from clathrin-mediated endocytosis (CME), which forms a vesicle inside the cell.

1986). These *vps* genes were initially categorized into three "classes"— A, B, and C—according to the morphology exhibited by the vacuole in yeast deficient with each gene (Banta et al., 1988). A fourth major class, denoted "class E," was also identified because of the distinctive endosome-derived compartment its members exhibited (Raymond et al., 1992). Through characterization of the trafficking of the vacuolar hydrolase carboxypeptidase S (CPS), it was demonstrated that transmembrane proteins can be sorted into the lumen of the vacuole via the MVB pathway (Odorizzi et al., 1998; Reggiori and Pelham, 2001). Two additional studies using a chimeric protein composed of CPS fused with the histidine biosynthesis enzyme His3 were used to select mutants defective in MVB sorting (taking advantage of the His minus phenotype exhibited by the His3-CPS fusion) (Katzmann et al., 2004; Odorizzi et al., 2003). By screening the various classes of *vps* mutants, Odorizzi et al. (1998) were the first to demonstrate that class E mutants were defective for CPS sorting to the lumen. Intriguingly, whereas His3-CPS sorting was attenuated with class E mutants, the trafficking of the soluble carboxypeptidase Y (CPY) was not. This suggested that the transport of integral membrane proteins followed a distinct route through the endosome (Odorizzi et al., 1998). Extensive biochemical and cell biological studies soon indicated that the class E proteins form distinct complexes that directly mediate this trafficking, and they were named the ESCRT complexes -I, -II, and -III (Asao et al., 1997; Babst et al., 1997, 2002a, 2002b; Katzmann et al., 2001; Shih et al., 2002) (Table 1). These complexes are all required for MVB

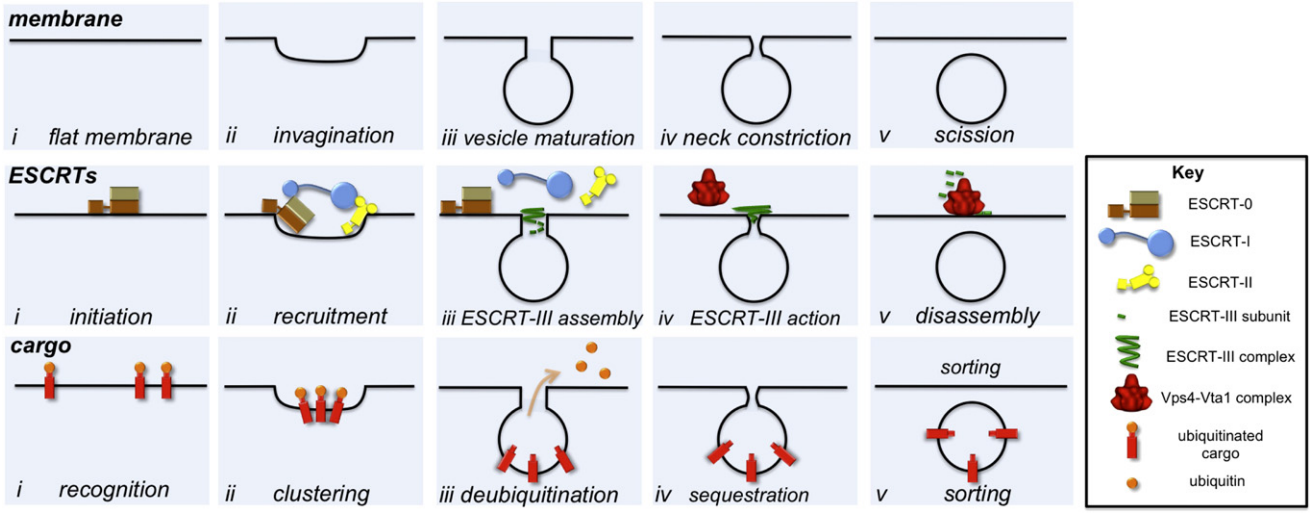
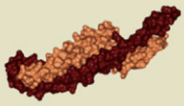
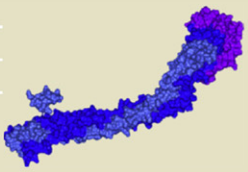
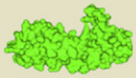


Figure 2. Perspectives of ESCRT-Mediated Vesicle Budding
The top view is from the membrane perspective, showing that there are five distinct stages of ILV budding. A flat membrane (i) is invaginated (ii) and matures into a vesicle that is still attached to the limiting membrane by a "neck" region (iii). The neck may undergo constriction (iv) and a scission event (v) to complete vesicle budding. The middle view is from the ESCRT protein perspective, in which ESCRT-0 initiates the pathway by engaging ubiquitinated cargo (i), until it is sequestered and sorted by ESCRT-III (iv and v). ESCRT-I and ESCRT-II complexes bind cargo and each other to create an ESCRT-cargo-enriched zone (ii). ESCRT-II nucleates ESCRT-III assembly (iii), which drives vesicle budding (iv) and is disassembled by the Vps4-Vta1 complex (v). The bottom view is from the cargo perspective, in which ubiquitinated cargo is first recognized by the ESCRT-0 ubiquitin-binding regions (i) and clustered by the ESCRT-0, -I, and -II complexes (ii). ESCRT-III assembly recruits deubiquitination machinery (iii) and packages cargo into the maturing vesicle (iv), which is finally sorted upon vesicle budding (v).

Table 1. Perspectives of the ESCRT Pathway

Complexes		Structure	Membrane Binding	Cargo Recognition	Intercomplex Interactions
Yeast	Human				
ESCRT-0					
Vps27	Hrs		with PtdIns3P via FYVE domain on Vps27	via UIM and VHS domains on both Vps27 and Hse1	to Vps23 (ESCRT-I) via a PTAP-like motif on Vps27 (ESCRT-0)
Hse1	STAM1/2				
ESCRT-I					
Vps23	Tsg101		weak electrostatics on N terminus of Vps37	via UEV domain on Vps23 and a novel UBD on Mvb12	to Vps27 (ESCRT-0) through UEV motif of Vps23 (ESCRT-I); to Vps36 (ESCRT-II) via the C terminus of Vps28 (ESCRT-I)
Vps28	hVps28				
Vps37	Vps37A,B,C				
Mvb12	hMvb12A,B				
ESCRT-II					
Vps36	EAP45		with PtdIns3P GLUE domain of Vps36	via GLUE domain on Vps36	to Vps28 (ESCRT-I) via the GLUE domain of Vps36 (ESCRT-II); to Vps20 (ESCRT-III) through C terminus of Vps25 (ESCRT-II)
Vps22	EAP30				
Vps25	EAP20				
ESCRT-III					
Vps20	CHMP6		myristoylation of Vps20; electrostatics on helix-1 of CHMP3	interacts with DUBs to deubiquitinate cargo	to Vps25 (ESCRT-II) through helix-1 of Vps20 (ESCRT-III); to Vps4 through C-terminal MIM domains present on all four ESCRT-III subunits
Snf7	CHMP4A,B,C				
Vps24	CHMP3				
Vps2	CHMP2A,B				
Vps4 Complex					
Vps4	SKD1				to MIM domains of ESCRT-III subunits via MIT domain
Vta1	LIP5				

The yeast and human ESCRT proteins are analyzed from the perspectives of their membrane interactions, their interactions with ubiquitinated cargo, and their interactions with one other. Please refer to text for references. PDB files: ESCRT-0 [3F1I], ESCRT-I [2P22], ESCRT-II [3CUQ], ESCRT-III [3FRT]; Vps4-Vta1 complex derived from Yu et al., 2008.

formation and are recruited from the cytoplasm to late endosomes in a sequential manner.

ESCRT-0

ESCRT-0 consists of two subunits, Hrs (*h*epatocyte growth factor-*r*egulated tyrosine kinase *s*ubstrate) and STAM1/2 (signal *t*ransducing *a*daptor *m*olecule1/2) (Vps27 and Hse1 in yeast) (Table 1). These subunits interact in a 1:1 ratio via coiled-coil GAT (*G*GAs *a*nd *T*om) domains (Asao et al., 1997; Prag et al., 2007). Although both subunits display structural similarities, one difference between them is a FYVE (*F*ab-1, *Y*GL023, *V*ps27, and *E*EA1) zinc finger domain on Hrs (Mao et al., 2000). The ability of the Hrs FYVE domain to bind phosphatidylinositol 3-phosphate, PtdIns3P, provides both membrane recruitment and endosomal specificity for the ESCRT-0 complex (Raiborg et al., 2001). Both subunits also bind ubiquitin, providing an additional targeting module that promotes their binding to cargo-enriched endosomes. The ability to bind both PtdIns(3)P and ubiquitin makes ESCRT-0 a coincidence detection module for initiating the ESCRT pathway at endosomes. This differs from ESCRT-I, which interacts only weakly with membrane through electrostatic interactions at the N terminus of Vps23 (Kostelansky et al., 2007). In fact, ESCRT-I recruitment to membrane is dependent upon ESCRT-0 in both metazoans and yeast (Bache et al., 2003; Katzmann et al., 2003; Lu et al., 2003). Thus, the recruitment of ESCRT-I by ESCRT-0 is essential for initiating MVB-dependent cargo sorting.

ESCRT-I

The first ESCRT complex to be described was ESCRT-I, identified originally in yeast as a heteromeric complex consisting of Vps23 (*t*umor *s*usceptibility *g*ene 101, or Tsg101 in mammals), Vps28, and Vps37 (Katzmann et al., 2001) (Table 1). Mvb12 (*m*ulti-*v*esicular *b*ody 12) was later identified as an additional subunit of ESCRT-I (Chu et al., 2006; Curtiss et al., 2007). Mammalian ESCRT-I similarly consists of Tsg101, Vps28, Vps37, and hMvb12, although additional isoforms of both Vps37 and hMvb12 exist (Bache et al., 2004; Bishop and Woodman, 2001;

Eastman et al., 2005; Morita et al., 2007; Stuchell et al., 2004). This is a theme repeated among the ESCRT complexes, as multiple isoforms of several mammalian ESCRT subunits exist. To our knowledge, the significance of multiple subunit isoforms is not yet known and may reflect tissue-specific variances among the ESCRT complexes. The crystal structure of the core yeast ESCRT-I complex reveals an elongated heterotetramer of ~20 nm in length, with three subunits intertwined into a long coiled-coil stalk with a globular head group (Kostelansky et al., 2007). ESCRT-I interacts with both ESCRT-0 and ESCRT-II, but at opposite ends of the complex. The interaction with ESCRT-0 occurs through the ubiquitin E2 variant domain (UEV), which corresponds to the N terminus of Vps23 and projects from the ESCRT-I stalk. The UEV binds to the PTAP-like motifs of the ESCRT-0 subunit Vps27/Hrs (Katzmann et al., 2003; Kostelansky et al., 2006), an interaction that is mimicked by the Gag protein of human immunodeficiency virus-1 (HIV-1) (Pornillos et al., 2003).

ESCRT-II

In addition to ESCRT-0, ESCRT-I also interacts with the ESCRT-II complex. ESCRT-II is a Y-shaped heterotetramer consisting of one subunit each of Vps22 (*E*LL-*a*ssociated *p*rotein of 30 kDa, or EAP30) and Vps36 (EAP45), and two subunits of Vps25 (EAP20) (Babst et al., 2002b; Langelier et al., 2006) (Table 1). Vps22 and Vps36 form the base of the Y and are each bound by one copy of Vps25, which forms the arms of the Y (Hierro et al., 2004; Teo et al., 2004). In yeast, ESCRT-II interacts with nanomolar affinity to ESCRT-I through an interaction between the GLUE (*G*RAM-*l*ike *u*biquitin-binding in *E*AP45) domain of Vps36 and the C terminus of Vps28 (Gill et al., 2007; Teo et al., 2006). Two NZF (*N*pl4-type *z*inc *f*inger) domains are inserted into the yeast GLUE domain; the first NZF binds to ESCRT-I, and the second NZF binds ubiquitin (Teo et al., 2006). The human GLUE domain has no NZF insertions but can still bind ubiquitin. Like ESCRT-0, ESCRT-II can also bind with high affinity to PtdIns(3)P via its GLUE domain (Slagsvold et al., 2005). Together with the ESCRT-0 FYVE domain, the GLUE and FYVE domains provide endosomal localization specificity by binding PtdIns(3)P, which is generated at endosomes through the class III PI3 kinase Vps34. Because the mammalian GLUE domain does not contain the NZF inserts required for the interaction with ESCRT-I, to our knowledge, the mammalian link between the ESCRT-I and ESCRT-II complexes remains uncharacterized. In contrast, the link between ESCRT-II and ESCRT-III has been defined—Vps25 binds to Vps20 with high affinity—revealing a critical role for ESCRT-II in initiating ESCRT-III complex formation.

ESCRT-III

Unlike the other ESCRT complexes, ESCRT-III does not form a stable, cytoplasmic complex, and attempts to crystallize an intact ESCRT-III complex have thus far failed. ESCRT-III consists of four core subunits: Vps20, Snf7, Vps24, and Vps2 (Babst et al., 2002a) (Table 1). In mammals these are denoted as *ch*arged *m*ultivesicular body *p*roteins (CHMPs) (CHMP6, CHMP4, CHMP3, CHMP2, and their isoforms respectively). The crystal structure of human Vps24, CHMP3, reveals an ~7 nm structure with five discernable helices; the core consisting of a hairpin formed by the first two helices (Bajorek et al., 2009b; Muzioł et al., 2006). A second structure of the "accessory" ESCRT-III protein Ist1 (*i*ncreased *s*alt *t*olerance-1) was recently solved and displays the same helical fold as CHMP3, suggesting that the architecture of the ESCRT-III subunits is generally conserved between subunits (Bajorek et al., 2009b).

ESCRT-III monomers do not localize to the endosome but exist in an autoinhibited "closed" state in the cytoplasm. To our knowledge, the exact mechanism for autoinhibition has yet to be determined; however, it is clear that it involves intramolecular interactions between the positively charged ESCRT-III N terminus and negatively charged C terminus (Shim et al., 2007; Zamborlini et al., 2006). Activation of ESCRT-III occurs when the ESCRT-II subunit Vps25 binds to Vps20, initiating ESCRT-III recruitment to the endosome and complex formation (Teo et al., 2004). Vps20 then recruits Snf7, which homo-oligomerizes. Snf7 polymers are thought to be capped by the recruitment of Vps24, which when mixed at a stoichiometric ratio of 10:1 with Snf7 in vitro can attenuate Snf7 homo-oligomerization (Saksena et al., 2009; Teis et al., 2008). Snf7 also recruits the ESCRT-III adaptor protein Bro1/Alix (*B*CK1-like *r*esistance to *o*smotic shock protein-1/*a*poptosis-*l*inked gene-2 *i*nteracting protein *X*), which stabilizes Snf7 filaments and recruits the deubiquitinating enzyme Doa4 (*d*egradation *o*f *a*lpha-4), necessary for cargo deubiquitination (Luhtala and Odorizzi, 2004; Odorizzi et al., 2003). Once at the endosome, Vps24 recruits Vps2, completing ESCRT-III complex assembly. Although the order of ESCRT-III subunit recruitment has been elucidated, to our knowledge, the exact stoichiometry of the ESCRT-III polymer remains uncharacterized and is a key point of emphasis for ongoing ESCRT studies.

In addition to the "core" ESCRT-III subunits, there are several "accessory" ESCRT-III subunits that govern ESCRT-III-Vps4 interactions. The most studied are Ist1, Did2 (*D*oa4-*i*ndependent *d*egradation-2), Vta1 (*V*ps *t*wenty-*a*ssociated-1), and Vps60. These ESCRT factors appear to modulate Vps4 function in three ways: by mediating Vps4 interactions with itself, with ESCRT-III subunits, and by promoting ATP hydrolysis. Vta1 promotes the ATPase activity of Vps4 and has been shown to form a supercomplex with oligomeric Vps4 at a 2:1 Vps4:Vta1 ratio (Azmi et al., 2006; Yu et al., 2008). The core of this supercomplex appears stable with a more flexible "cap" at one end. Vta1 also interacts directly with ESCRT-III subunits, Vps60 in particular, via two MIT domains (*m*icrotubule-*i*nteracting and *t*rafficking) at its own N terminus that bind to MIMs (*M*IT-*i*nteracting *m*otifs) on the ESCRT-III subunits. Thus, Vta1 may function as an adaptor bridging Vps4 and ESCRT-III (Azmi et al., 2008; Shiflett et al., 2004). Bro1/Alix is also an important ESCRT-III accessory subunit and binds to the extreme C terminus of Snf7. Loss of Bro1/Alix affects ESCRT cargo sorting in yeast (Odorizzi et al., 2003). It is thought to both stabilize Snf7 oligomers and recruit the deubiquitinating enzyme Doa4.

Vps4-Vta1 Complex

Once assembled, the ESCRT-III complex requires energy to disassociate from the membrane. This energy is provided by the class I AAA (*A*TPase *a*ssociated with various cellular *a*ctivities) ATPase Vps4 (Babst et al., 1998). Vps4 is a multimeric mechanoenzyme that binds ESCRT-III subunits via a N-terminal MIT domain that recognizes C-terminal MIMs present in the ESCRT-III subunits (Babst et al., 1997; Scott et al., 2005b) (Table 1). It exists in vitro as a monomer or dimer in the nucleotide-free or ADP-bound state but multimerizes into a stable dodecamer of two hexameric rings and a supercomplex with Vta1 when fully assembled (Babst et al., 1997; Scott et al.,

2005a; Yu et al., 2008). ATP hydrolysis is promoted by multimerization and association with ESCRT-III subunits and is necessary for removal of ESCRT-III subunits from the membrane because expression of a hydrolysis mutant Vps4 E233Q leads to the accumulation of hyperoligomeric ESCRT-III subunits on the endosome and a class E phenotype (Babst et al., 1998).

AAA ATPases are involved in a wide array of cellular processes, including membrane trafficking and fusion, DNA replication, proteolysis, and cytoskeletal reorganization (Barends et al., 2010; Neuwald et al., 1999; Striebel et al., 2009). However, there are common themes shared between AAA ATPases that provide insight into Vps4 function. All share one or two AAA domains of ~250 amino acids that bind ATP in most members. Most multimerize and form hexameric rings. Several AAA ATPases alter substrate conformation, including the heat shock-related protein ClpX (*c*aseino*l*ytic *p*eptidase *X*) that has been shown to mediate substrate unfolding (Weber-Ban et al., 1999). Protein unfolding is thought to be achieved by capturing and "threading" proteins through a narrow pore in the core of the AAA oligomer (Reid et al., 2001; Weber-Ban et al., 1999). It has been suggested that the Vps4-Vta1 complex mediates ESCRT-III disassembly in this manner. Here, Vps4-Vta1 binds ESCRT-III subunits and threads them through a central pore in an ATP-dependent process. Although only a model, mutation of residues within the Vps4-Vta1 oligomer pore leads to defects in HIV-1 budding (Gonciarz et al., 2008). If this is truly how ESCRT-III is disassembled, then, to our knowledge, how ESCRT-III subunits are refolded after dissociation remains to be elucidated, although ClpX substrate MuA is thought to spontaneously refold following ClpX activity (Burton et al., 2001). Notably, the Vps20 and Snf7 MIMs (MIM2 type) appear to bind a different area of the Vps4 MIT than the Vps2 and Vps24 MIMs (MIM1 type), and all bind with low affinity, suggesting that multiple ESCRT-III subunits must interact with Vps4 for stable recruitment (Shestakova et al., 2010).

Vps4 interacts with the accessory ESCRT-III subunits to further modulate its activity. As discussed above, Vta1 forms a heteromeric complex with Vps4. The presence of MIT domains on Vta1 and on each Vps4 subunits means that the Vps4-Vta1 supercomplex contains up to 24 MIT domains, allowing it to interact with multiple ESCRT-III subunits simultaneously. Did2 and Ist1 form a complex in yeast and together interact with both Vps4 and ESCRT-III (Rue et al., 2008). This complex may function as an endosomal anchor for the Vps4 oligomer, because Vps4 lacking its MIT domain still localizes to endosomes but is redistributed to the cytoplasm in yeast lacking Did2 (Shestakova et al., 2010).

Cargo: ESCRT-Mediated Cargo Recognition and Sorting

Cargo Engagement

A key characteristic required by any cargo-sorting machinery is the ability to recognize and engage cargo. This review now switches perspectives from the ESCRT proteins to the ubiquitinated cargo that they bind. Accordingly, ESCRT complexes have several distinct ubiquitin-binding motifs (Bilodeau et al., 2002; Hirano et al., 2006; Mizuno et al., 2003; Pornillos et al., 2002; Raiborg et al., 2002; Shields et al., 2009; Shih et al., 2002; Slagsvold et al., 2005). Both subunits of the earliest ESCRT complex, ESCRT-0, bind ubiquitin in numerous ways. This highlights ESCRT-0 as a cargo-recognition module. Hrs binds ubiquitin via a double-sided ubiquitin-interacting motif (UIM) and a VHS (Vps27 Hrs STAM) domain (Hirano et al., 2006) (Ren and Hurley, 2010). The yeast homolog of Hrs, Vps27, also contains a VHS domain that binds ubiquitin (Table 1). Interestingly, Vps27 contains two UIMs in tandem instead of a double-sided UIM (Bilodeau et al., 2002). STAM1/2 and its yeast homolog Hse1 contain both a UIM and a VHS domain for binding ubiquitin (Bilodeau et al., 2002; Fisher et al., 2003; Mizuno et al., 2003). Although the ESCRT-0 heterodimer forms GAT domains that have been shown to bind ubiquitin in other proteins, to our knowledge, direct binding of ubiquitin to the ESCRT-0 GAT domain has not been observed (Prag et al., 2007; Ren et al., 2009). However, ESCRT-0 clearly contains several ubiquitin-binding domains (approximately five in yeast), although it is unclear if this is primarily to engage several cargoes simultaneously, to bind tightly to a cargo through avidity interactions, or even bind with high affinity to polyubiquitinated cargo (Ren and Hurley, 2010).

In addition to its ubiquitin-binding motifs, ESCRT-0 may engage cargo through its interactions with the clathrin vesicle machinery at the endosome. Vps27/Hrs binds clathrin heavy chain via its clathrin box motif (Raiborg et al., 2001). STAM contains a canonical clathrin-binding motif, but it is unclear if it binds clathrin in vivo (McCullough et al., 2006). The interaction between ESCRT-0 components and clathrin results in microdomains of flat clathrin lattices, ESCRT-0, and ubiquitinated cargo that appear important for cargo sorting (Raiborg et al., 2006; Sachse et al., 2002).

ESCRT-I and ESCRT-II also contain ubiquitin-binding domains (UBDs). Tsg101 and hMvb12 and their corresponding yeast homologs both bind ubiquitin. In addition, Tsg101 and Vps23 contain a catalytically inactive UEV (Pornillos et al., 2002). Recently, a novel UBD was identified on yeast Mvb12 and human Mvb12A (Shields et al., 2009; Tsunematsu et al., 2010) (Table 1). Thus far, UBDs have been identified on only one subunit of ESCRT-II, Vps36 (EAP45). In yeast Vps36, the GLUE domain contains a NZF motif insertion that binds ubiquitin (Alam et al., 2004). The GLUE domain of the mammalian homolog EAP45 also binds ubiquitin, although the NZF motifs are not present (Slagsvold et al., 2005) (Table 1). Notably, to our knowledge, no UBDs have been identified on the ESCRT-III subunits. This is consistent with a role for ESCRT-III primarily in vesicle budding and scission, and not in cargo recognition, and highlights the division of labor between the ESCRT complexes. Whereas the "early" ESCRT-0, -I, and -II complexes display direct ubiquitin binding and high phosphoinositide specificity, ESCRT-III does not require these characteristics to achieve vesicle formation at sites already enriched with cargo.

Deubiquitination

Before delivery to the vacuole/lysosome, cargo must be deubiquitinated. This event appears to occur just prior to packaging of the cargo into the ILV. In mammals AMSH (*a*ssociated *m*olecular with *SH3* domain of STAM), a ubiquitin isopeptidase, interacts with both ESCRT-0 and ESCRT-III (McCullough et al., 2006) and is responsible for the deubiquitinating step (Kyuuma et al., 2007). A second mammalian ubiquitin isopeptidase Y (UBPY) also interacts with ESCRT-0 and is critical in maintaining cellular ubiquitin levels (Row et al., 2006). Unlike AMSH, which only recognizes Lys-63-linked polyubiquitin chains, UBPY deubiquitinates both Lys-63 and Lys-48 polyubiquitin chains (Row et al., 2006). In yeast, Doa4 deubiquitinates

cargo, and its loss depletes ubiquitin pools and alters ubiquitin homeostasis because of its numerous non-ESCRT functions (Amerik et al., 2000; Swaminathan et al., 1999). Thus, ESCRTs have an important role in recruiting the deubiquitinating machinery required to remove ubiquitin from cargo and maintain cellular ubiquitin pools.

In addition to binding ubiquitin, ESCRT proteins can themselves be ubiquitinated. The ESCRT-0 subunit Vps27/Hrs is monoubiquitinated (Polo et al., 2002), although this ubiquitination is not essential for Vps27 function, and to our knowledge, its physiological significance is unknown (Stringer and Piper, 2011). However, Hrs ubiquitination can be inhibitory because the intramolecular interaction of the Hrs UIM and ubiquitin prevents the binding of ubiquitinated cargo (Hoeller et al., 2006). Cargo fused to a single ubiquitin can successfully be sorted by an ESCRT pathway with a deubiquitinating enzyme fused to ESCRT subunits, reinforcing the idea that ESCRT subunit ubiquitination may not be essential for MVB sorting (Stringer and Piper, 2011).

In addition to deubiquitinating cargo, AMSH is responsible for removing the ubiquitin from Hrs (Sierra et al., 2010). Similarly, the ESCRT-I subunit Tsg101 is ubiquitinated by the E3-ubiquitin protein ligase Mahogunin, the disruption of which affects endosome to lysosomal trafficking (Kim et al., 2007). This observation is intriguing because ubiquitination of Tsg101 by another E3 ligase, Tal, is inhibitory and disrupts both MVB formation and viral budding (Amit et al., 2004).

In summary, from the perspective of the cargo, the ESCRT pathway is subdivided into early stages where cargo is recognized and potentially clustered (ESCRT-0, -I, and -II) until it is corralled by the downstream ESCRT-III complex, which lacks ubiquitin binding, but is able to couple ESCRT-III assembly with cargo deubiquitination and vesicle budding.

ESCRT-Mediated Membrane Deformation and Scission

Retroviral Budding

This review now moves to focus on the ESCRT pathway from the perspective of the lipid membrane it can deform. ESCRT-mediated MVB biogenesis requires membrane deformation and scission to generate an ILV. When ESCRTs were identified as essential factors in the budding of the HIV-1, it became clear that ESCRT proteins were potentially direct mediators of the membrane remodeling necessary for vesicle or viral particle budding (Garrus et al., 2001). Since then, the ESCRTs have been implicated in the replication of many viruses. Recent reviews have examined in detail the roles of ESCRT proteins in viral budding (Carlton, 2010; Chen and Lamb, 2008). For the purpose of this review, we will focus on the extensively studied role of ESCRTs in HIV-1 budding from the plasma membrane as a model viral system.

Perhaps the most striking difference between MVB biogenesis and viral budding is the differential requirement of ESCRT components in these processes. Unlike MVB formation, HIV-1 budding does not require ESCRT-0, ESCRT-II, or the ESCRT-III subunit hVps20 (CHMP6) (Langelier et al., 2006). In fact, overexpression of a C-terminal fragment of Hrs inhibits HIV particle release, likely due to its sequestration of Tsg101 (Bouamr et al., 2007). Tsg101 mediates HIV-1 budding by binding to the L (late) domain of the viral Gag protein (Garrus et al., 2001; VerPlank et al., 2001). Similar to HIV-1, other viruses contain L-domain proteins that interact directly with the ESCRT machinery (Carpp et al., 2011; Martin-Serrano et al., 2001; Urata et al., 2007; Wirblich et al., 2006). They function in combination with other ESCRT proteins to recruit ESCRT-III and Vps4 to sites of viral budding. Interestingly, the ubiquitination of Gag can also enhance its interaction with the ESCRTs by providing additional binding sites for Tsg101 (Garrus et al., 2001). Accordingly, a role for Nedd4 and the ubiquitin ligase machinery has been identified in the release of enveloped viruses (Strack et al., 2000).

Because ESCRT-II is not required for HIV budding, a question arises as to how ESCRT-III is recruited to sites of viral release. Bro1/Alix is an attractive candidate because it can bind ESCRT-I and ESCRT-III, and localizes to viral bud sites (Odorizzi et al., 2003). Indeed, live cell imaging of EIAV viral budding from *HeLa* cell plasma membranes indicates that GFP-Alix is recruited to viral bud sites very early along with the viral Gag protein (Jouvenet et al., 2011). Future studies will be necessary to dissect how Alix bridges ESCRT-I and ESCRT-III and functions in viral budding.

Cytokinesis

The discovery that ESCRT proteins are necessary for cell abscission, the final stage of cytokinesis in which the cell midbody is constricted and severed, clearly establishes the ESCRTs as a multipurpose machine that can execute numerous topologically equivalent membrane-deforming processes. Cytokinesis requires membrane scission, and the creation of membrane curvature that is topologically consistent with the curvatures needed in MVB sorting and viral budding. Thus, the ESCRTs provide an intuitive mechanism as executioners of the abscission step. However, this link was not always obvious. In addition to the ESCRTs, numerous membrane-trafficking proteins are required for cell division, including dynamin, SNAREs, exocyst components, and Rab proteins (Gromley et al., 2005). Furthermore, both endocytosis and exocytosis are required for cytokinesis, likely because new membrane must be delivered to the midbody during cleavage furrow progression (Danilchik et al., 1998; Gerald et al., 2001).

What then is the role of membrane-trafficking proteins in cell division? Until recently, the leading model was that endosomes were recruited and clustered at the midbody as cytokinesis progressed, then underwent a massive round of homotypic fusion as well as heterotypic fusion with the plasma membrane (Danilchik et al., 1998; Gromley et al., 2005). This would have the net effect of adding membrane to the midbody as it was contracted by the actomyosin ring. The narrow neck remaining after this ingression would finally undergo a fission event, thus completing abscission.

Tsg101 and Bro1/Alix were the first ESCRT proteins found to localize to late-stage midbodies (Carlton and Martin-Serrano, 2007; Spitzer et al., 2006). However, because endosomes localize to the midbody, it was assumed that ESCRTs function in their capacity as MVB-sorting machines during cytokinesis. However, two findings argue against this. One is that Tsg101 and Bro1/Alix are recruited to the midbody by Cep55, a multimeric cell division protein essential for a late stage in cell division (Carlton et al., 2008; Carlton and Martin-Serrano, 2007). Importantly, Cep55 depletion prevents Tsg101 and Bro1/Alix recruitment to the midbody, while not affecting viral budding, demonstrating that these two processes are distinct (Carlton and Martin-Serrano, 2007). The second is that Crenarchaeota of the genus *Sulfolobus* uses ESCRT-III and Vps4

orthologs directly in cytokinesis (Lindås et al., 2008), suggesting an ancient role for these proteins in cell division. Furthermore, a Vps25-like protein CdvA can interact with and recruit ESCRT-III-like subunits to membranes in *Sulfolobus* (Samson et al., 2011).

Thus, an emerging model is that ESCRT-I and Bro1/Alix are recruited to the midbody by directly binding Cep55 and in turn recruit ESCRT-III to commit the final scission event in cytokinesis (Carlton and Martin-Serrano, 2007). Although direct evidence for ESCRT-III involvement in the actual scission event of abscission is lacking, depletion of Vps4 or expression of dominant negative hVps24 (CHMP3) arrests late cytokinesis (Dukes et al., 2008). Recent studies have also demonstrated that CHMP4B and Tsg101 localize to two distinct rings at opposing ends of the cell midbody (Elia et al., 2011; Guizetti et al., 2011). Using electron tomography, Guizetti et al. (2011) recently visualized protein filaments that spiral around the constriction zone of a dividing cell. ESCRTs were required for the formation of these filaments, and CHMP4b colocalized with them (Guizetti et al., 2011). It is hypothesized that constriction or other conformational changes within these rings drive the final stages of abscission. This ring formation is a distinct late event after cleavage furrow progression because the midbody will form and progress to late stages before being arrested in ESCRT-I or -III depleted cells. Ist1 also localizes to the midbody and is essential for abscission (Agromayor et al., 2009; Bajorek et al., 2009a). How ESCRT-mediated scission is coordinated with actomyosin constriction and potential SNARE-mediated fusion at the midbody is still not understood.

Recent insight into how microtubule disassembly at the midbody occurs demonstrates a second important role for the ESCRTs in cytokinesis. The AAA-ATPase spastin is recruited to the midbody by ESCRT-III subunit CHMP1B and subsequently severs microtubules as abscission progresses (Yang et al., 2008). This suggests that ESCRT-III can recruit at least two AAA ATPases to sites of scission.

Themes of Vesicle Budding

Thus, at present all well-characterized ESCRT-mediated processes involve membrane deformation (i.e., budding) and scission. Furthermore, this membrane deformation is topologically consistent between processes in that it produces membrane curvature that pushes away from the cell cytoplasm (Figure 2). This is in contrast to "classical" budding events like COP-I, COP-II, and clathrin vesicle formation, which invoke vesicle budding into the cytoplasm (Doherty and McMahon, 2009; Lee et al., 2005; Rothman and Wieland, 1996; Shimoni and Schekman, 2002). "Classical" vesicle formation involves a cytoplasmic protein coat that encapsulates the vesicle, adaptor proteins that help to sequester cargo and promote vesicle maturation, and a scission mechanism that liberates the vesicle from the source membrane. During dynamin-mediated endocytosis, this scission event is promoted by the formation of a narrow "neck" of extreme curvature on which helical dynamin oligomers encircle and potentially constrict to achieve scission, although the exact nature of this scission event is still contested (Hinshaw and Schmid, 1995). Thus, from the membrane's perspective, "classical" vesicle budding can be divided into five stages: a flat membrane (1) is invaginated (2), forming a vesicle and neck region (3) that undergoes constriction (4) and scission (5) (Figure 2). These stages appear to be tightly regulated as evidenced by the extreme uniformity of vesicle size for a particular vesicle type.

In ESCRT-mediated vesicle formation, ESCRT complexes cannot coat the vesicle exterior because they are segregated in the cytoplasm away from the luminal vesicle by the limiting membrane of the endosome. It is possible that ESCRT-III could coat the vesicle interior during vesicle maturation, but if so the ESCRTs are efficiently removed prior to scission because they are not consumed in the reaction (Babst, 2005; Wollert and Hurley, 2010). Furthermore, because the vesicle neck and scission mechanism is topologically inverted, the ESCRTs cannot encircle any neck that forms (Figure 2). Despite this, the membrane must undergo analogous stages of progression to achieve ILV formation. This is supported by the observation that ESCRT-mediated vesicle budding is highly regulated because endosomal ILVs retain a consistent diameter (Teis et al., 2010). Thus, "classical" and ESCRT-mediated vesicle budding events are conceptually similar yet mechanistically distinct.

Analyzing ESCRT-Mediated Vesicle Budding from a "Classical" Perspective

Because ILV formation is topologically distinct from "classical" vesicle formation, how then is ESCRT-mediated vesicle formation achieved? Recent studies have defined a key role for the ESCRT-III complex in particular as a membrane deformation and scission machine (Hanson et al., 2008; Saksena et al., 2009; Wollert and Hurley, 2010; Wollert et al., 2009). Although these studies partially reconstituted ESCRT-III scission activity in vitro, the mechanism by which ESCRT-III achieves this remains elusive. Furthermore, it is unclear if there is a division of labor between the ESCRT complexes to mediate the stages of membrane deformation and scission in vivo. Using giant unilamellar vesicles (GUVs), studies by Wollert et al. (2009) demonstrated that ESCRT-III subunits alone were sufficient to bud and release ILVs in vitro. However, ESCRTs -I and -II could deform the membrane, although scission was not observed (Wollert and Hurley, 2010). The observation that ESCRTs -I and -II can generate membrane curvature in vitro is intriguing, but the mechanism by which they do this is also unclear. The membrane-binding portions of these complexes are small. Vps37 contains a small positively charged N terminus that binds acidic liposomes, but this N terminus is not required for ESCRT-I function in vivo (Kostelansky et al., 2007). ESCRT-II binds PtdIns(3)P with high affinity through the Vps36 GLUE domain, and can bind negatively charged lipids electrostatically via a small helix on the N terminus of Vps22, but it is unclear how these domains can generate membrane curvature (Im and Hurley, 2008).

Despite their different topologies, "classical" vesicle budding mechanisms may provide additional insight into ESCRT-mediated vesicle budding. Recent models suggest that dynamin mediates scission by forming an oligomeric scaffold around the vesicle neck, as well as potentially penetrating the lipid bilayer. This has the net effect of promoting fission at the vesicle neck (Ramachandran et al., 2009; Roux et al., 2006; Stowell et al., 1999). In yeast, the membrane-sculpting potential of the heterodimeric Bin/Amphiphysin/Rvs (BAR) domain-containing complex Rvs161/Rvs167 promotes scission by generating

a highly curved vesicle neck that is stressed by the pulling forces of actin polymerization (Kaksonen et al., 2005). BAR superfamily proteins can promote vesicle budding by binding electrostatically to negatively charged membranes and bending them to match the intrinsically bent shape of their dimers (Gallop and McMahon, 2005; Henne et al., 2007; Peter et al., 2004). N-BAR domain-containing proteins can also generate high membrane curvature via their N-terminal amphipathic helices that penetrate the lipid bilayer (Peter et al., 2004). In COP vesicle budding, Sar1 and possibly Arf1 promote vesicle formation by membrane insertion of protein residues (Lee et al., 2005; Long et al., 2010; Lundmark et al., 2008). Thus, in other protein-mediated budding systems, vesicle formation is promoted by at least three distinct mechanisms: (1) individual protein scaffolding via electrostatic protein-membrane interactions, (2) oligomeric scaffolding, and (3) membrane insertion of hydrophobic residues. Can these mechanisms be applied to ESCRT-mediated ILV formation?

Snf7 Oligomer-Mediated Vesicle Budding

ESCRT-III subunits share at least two features with proteins that promote "classical" vesicle budding: they bind to lipid membrane electrostatically via their positively charged N termini, and some subunits can oligomerize (Hanson et al., 2008; Muzioł et al., 2006; Teis et al., 2008). Like dynamin, Snf7 forms oligomeric assemblies (Ghazi-Tabatabai et al., 2008; Hanson et al., 2008; Pires et al., 2009). In vitro or when overexpressed in vivo, these appear in different forms as rings, straight filaments, or spirals of varying diameter. Although Snf7 oligomers are the best characterized, filaments of Vps24 and the Vps24-Vps2 subcomplex have also been observed (Ghazi-Tabatabai et al., 2008; Lata et al., 2008). Hanson et al. (2008) observed striking membrane deformations associated with oligomerized Snf7, leading to the hypothesis that the assembly of Snf7 oligomers promotes membrane bending and potentially vesicle formation.

A model explaining how Snf7 oligomers mediate ILV budding must address the observation that ESCRT-II is Y-shaped and has two Vps25 "arms" that can nucleate two Snf7 filaments. In one model, each Vps25 arm binds a Vps20 subunit, which in turn nucleates two Snf7 homo-oligomers that extend out to "encircle" cargo in a ring or spiral (Figure 3iv). Strikingly, mutation of the Vps25-binding sites of Vps36 or Vps22 to generate "one-armed" ESCRT-II does not inhibit Snf7 filament formation but does halt ILV formation (Teis et al., 2010). Thus, a "two-armed" ESCRT-II is necessary for vesicle formation in vivo, and points to a carefully regulated ESCRT-III superstructure of defined size and architecture. This is consistent with the fact that ILVs exhibit a consistent diameter (~25 nm in yeast) and that ESCRT-III is sized as an oligomer of ~450 kDa in yeast extracts. Interestingly, overexpression of Snf7 leads to hyperpolymerization of ESCRT-III into a >600 kDa structure, and the formation of ILVs with varied but significantly larger diameter (up to 360 nm) (Teis et al., 2010).

Because hyperoligomeric ESCRT-III is associated with irregular ILV diameter, how is ESCRT-III oligomer size regulated? The Snf7 oligomer is "capped" by Vps24 and Vps2, which are recruited after Snf7 oligomerization and function to: (1) halt Snf7 filament progression, and (2) recruit Vps4-Vta1 for ESCRT-III complex disassembly. Snf7 oligomerization can also be manipulated by modulating the expression of the ESCRT-III protein Bro1/Alix. Bro1 overexpression enhances Snf7 oligomer stability and leads to ILV invaginations within the endosome that do not undergo scission, indicating that ESCRT-III disassembly is required prior to ILV formation (Wemmer et al., 2011). Notably, this is in contrast to in vitro studies, where vesicle release occurred prior to Vps4-mediated ESCRT-III disassembly (Wollert et al., 2009).

Thus, regulated Snf7 oligomerization represents one model of ESCRT-III-mediated ILV budding. In it, membrane deformation is directly coupled to Snf7 filament assembly and "capping." Snf7 oligomerization also encircles cargo already clustered by the "upstream" ESCRT complexes so it can be deubiquitinated and corralled into the maturing vesicle. Here, the formation of a Snf7 ring acts as a physical barrier preventing transmembrane cargo from escaping the vesicle. This is evidenced by the fact that loss of Snf7 promotes retrograde trafficking of cargo back to the plasma membrane, but loss of Vps24 does not (Teis et al., 2008). However, this model fails to completely provide a mechanism for membrane scission. Snf7 oligomers observed in vivo appear as flat spirals and require Snf7 truncation or the coexpression of dominant-negative Vps4 to induce membrane curvature (Hanson et al., 2008). Furthermore, the spirals are ~100–300 nm in diameter, and it is unclear how they would allow a narrow ILV neck to form. One possibility is that Snf7 oligomers naturally curve to favor an ideal diameter, and, as oligomerization progresses, filaments narrow to an optimal diameter that is terminated by the "cap" Vps24-Vps2 subcomplex. This narrowing may constrict the ILV neck enough to promote scission.

Snf7 Oligomerization and Membrane Buckling

Another ESCRT-III oligomeric model attempts to explain how ESCRT-III can generate membrane buckling and subsequent fission. In this "buckling ring" model originally proposed by Lenz et al. (2009), ESCRT-III rings have an intrinsic optimal radius but accumulate around one another in varying radii due to the high affinity ESCRT-III subunits have for one another and the membrane. The membrane responds by deforming into an inverted tube to relax the tension on the rings so they can readjust to their preferred diameter (Lenz et al., 2009). The result is an elongated tube containing numerous ESCRT-III rings of preferred diameter contacting each other and the membrane.

This provides a mechanism for how ESCRT-III generates membrane curvature but also does not provide an intuitive mechanism for scission. This model places ESCRT-III rings down the length of an elongated ILV tube but fails to explain how they would be removed prior to budding.

The Dome Model

An alternative model for ESCRT-III-mediated vesicle formation implicates the Vps24-Vps2 subcomplex as its central mediator. It is based on studies by Lata et al. (2008) who observed in vitro cylinders composed of stacked Vps24-Vps2 rings or spirals that bind to liposomes, suggesting that their membrane-binding surface faces the outside of the cylinder. These cylinders form tapered ends of decreasing diameter, terminating in a protein hemisphere or "dome" (Figure 3v). This has given rise to the "Dome Model" of ESCRT-III-mediated scission where sequential ESCRT-III rings or spirals of decreasing diameter stack inside the neck of the forming ILV and are capped by a Vps24/Vps2 hemisphere (Fabrikant et al., 2009).

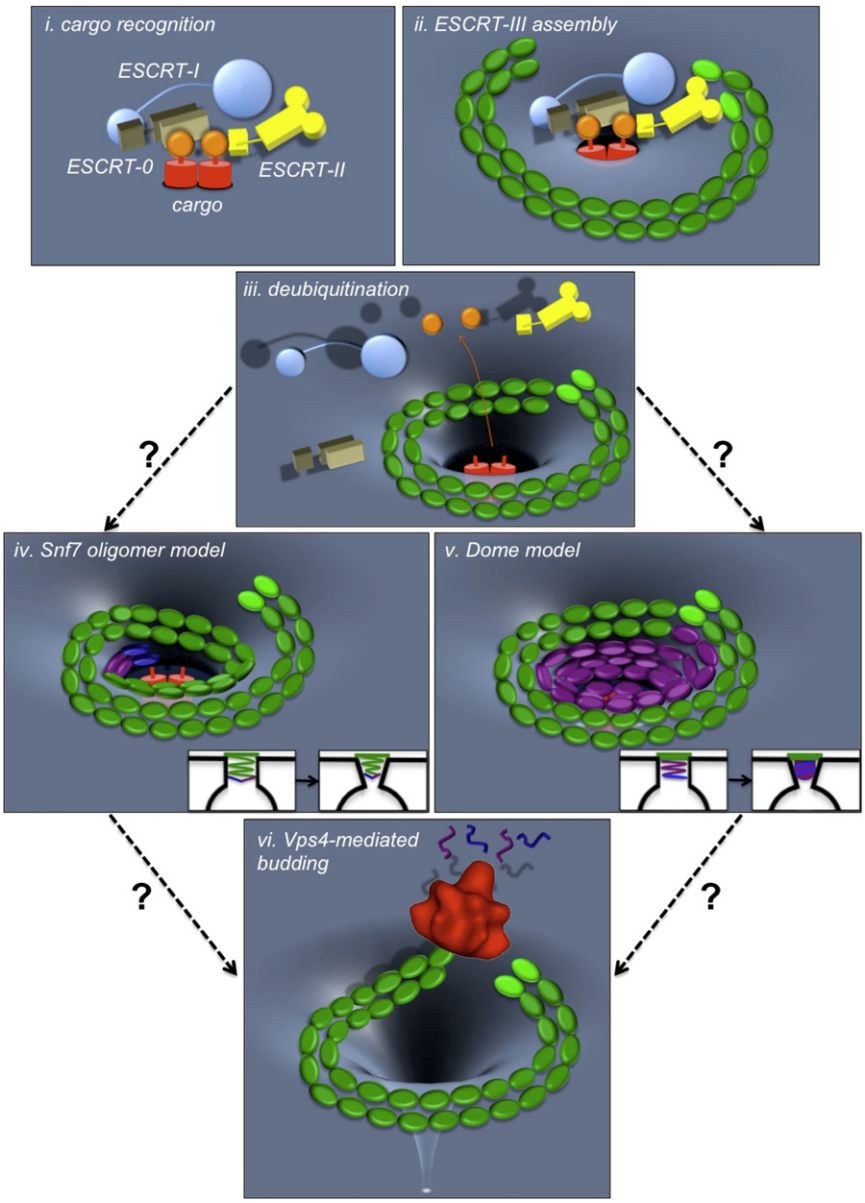


Figure 3. Models of ESCRT-Mediated Vesicle Budding

The first three panels represent "upstream" ESCRT complex recognition of ubiquitinated cargo (i), the initiation of ESCRT-III assembly by ESCRT-II (ii), and deubiquitination of the cargo (iii). From there, two different models of ESCRT-III-mediated vesicle maturation are presented. The Snf7 oligomerization model (iv) indicates that vesicle formation is a consequence of controlled Snf7 homo-oligomerization (green) initiated by Vps20 (light green) and "capping" by Vps24 (purple) and Vps2 (dark blue). In the Dome model (v), Snf7 filaments define a "zone" where rings of Vps24 and Vps2 form into a protein hemisphere, which drives vesicle budding. Both models may be dependent on Vps4 for neck constriction (vi), which drives vesicle budding.

Importantly, membrane attachment to the dome mediates constriction of the ILV neck. Constriction is therefore dictated by the protein affinity for the membrane, generating a highly curved membrane dome that buckles to facilitate scission. Although Vps24-Vps2 filaments drive vesicle scission, they are recruited by Vps20 and the Snf7 oligomer that localize to sites of ILV formation prior to dome formation. Thus, the "Dome Model" is consistent with the late recruitment of Vps24 and Vps2 seen in ESCRT-III assembly (Saksena et al., 2009; Teis et al., 2008).

One critique of the "Dome Model" is that it does not address why the Vps24-Vps2 subcomplex was not necessary for ILV budding into GUVs (Wollert and Hurley, 2010). Likewise, overexpression of Snf7 and mutant Vps4 was responsible for the dramatic plasma membrane deformations observed by Hanson et al. (2008), suggesting that the Vps24-Vps2 complex is not required for Snf7-induced membrane deformations in vivo.

Vps4 and Membrane Scission

Although Vps4 is necessary for ESCRT-III disassembly and MVB sorting, it is unclear if it plays a direct role in ILV scission. There are two general hypotheses for its role. We favor the idea that the Vps4-Vta1 complex engages ESCRT-III following its assembly and, like a mechanical motor, helps generate the constrictive force necessary for scission at the ILV neck. In this model, Vps4-Vta1 oligomers bind ESCRT-III subunits and mediate the stepwise disassembly of the ESCRT-III complex (Figure 3vi). This stepwise disassembly could generate force along the ESCRT-III polymer and mediate neck constriction as the polymer shrinks into smaller and smaller rings, similar to a tightening "purse string" (Saksena et al., 2009). Disassembly could also potentially generate a "sliding" mechanism where Snf7 filaments move past one another, creating constrictive force. Alternatively, the Vps4-Vta1 oligomer could engage multiple ESCRT-III subunits simultaneously and mediate concerted disassembly of the ESCRT-III complex. The drastic removal of the ESCRT-III complex from the neck of the budding ILV may destabilize the highly curved membrane, leading to buckling and scission (similar to the "buckling model" above). A similar model for vesicle scission has been proposed for dynamin, where dynamin oligomers spiral around a membrane tubule but release their hold just prior to scission (Bashkirov et al., 2008). In all models, the late recruitment of Vps4 ensures that ESCRT-III is not prematurely disassembled. The late recruitment of Vps4 is supported by the ordered assembly of ESCRT-III because Vps4 avidity for the ESCRT-III subunits will be greatest only after the complex is fully assembled (Teis et al., 2008).

An alternative model suggests that Vps4 does not mediate ILV budding directly but rather serves only to "recycle" ESCRT-III subunits after scission has occurred (Wollert et al., 2009). This would place the ATP hydrolysis reaction after the scission event, similar to the role of the AAA-ATPase NSF (N-ethylmaleimide sensitive fusion protein) in recycling the SNARE complex after membrane fusion. This is in contrast to other vesicle budding events like dynamin-dependent endocytosis, where the hydrolysis of GTP causes the dynamin oligomer to change conformations and mediate scission. Here, Vps4 would function primarily to remove ESCRT-III subunits from the membrane and potentially "re-set" them into their autoinhibited conformation. This "closed" conformation places the C terminus in close proximity with the α-helical hairpin formed by the first two helices of the ESCRT-III structure (Bajorek et al., 2009b; Muzioł et al., 2006; Zamborlini et al., 2006). It is intriguing that Vps4 binds the C-terminal MIM motifs, indicating that it could mediate C-terminal conformational changes.

Notably, both the Snf7 oligomeric model and Vps24-Vps2 "Dome" model could be dependent on Vps4 for scission. Vps4-mediated disassembly could provide the constrictive force for Snf7-mediated scission (Saksena et al., 2009). In the "Dome" model, the Vps24-Vps2 hemisphere causes the ILV membrane neck to taper, bringing the membrane into close proximity for spontaneous fission. However, one could speculate that the vesicle neck may be stabilized by its high affinity for Vps24-Vps2 dome. Here, removal of the protein dome by Vps4 could mediate membrane buckling and scission.

Lipid Composition and ILV Budding

Careful consideration should also be placed on the role of lipids in ILV formation. Although in vitro evidence suggests a prominent role for the ESCRTs in vesicle budding, several studies indicate that specific lipids are key mediators of vesicle formation. Sphingomyelin can form liquid ordered domains with cholesterol and, when converted to ceramide by sphingomyelinases, can generate membrane deformations that bud ILVs in vitro (Trajkovic et al., 2008). Lysobisphosphatidic acid (LBPA) has also been shown to generate multivesicular liposomes in a pH-dependent manner, although it is unclear if this lipid exists in yeast (Matsuo et al., 2004). It is highly likely that the clustering of protein complexes like the ESCRTs into small cargo-enriched regions on the endosome drives some lipid ordering. This ordering may reinforce ESCRT-mediated membrane deformation, although the detailed mechanism of this remains to be elucidated.

Summary and Synthesis

Thus, to date, there are two broad models for ESCRT-III-mediated ILV formation, although variants of each of them exist. In one, Snf7 oligomers encircle cargo and recruit "capping" proteins Vps24 and Vps2, which regulate Snf7 oligomer size and recruit Vps4-Vta1. Vesicle formation is thus a consequence of controlled Snf7 oligomerization. In the other model, a Vps24-Vps2 "dome" forms within the neck of a budding ILV, and its electrostatic affinity for membrane drives the constriction of the ILV neck to promote scission. Emphasis is thus placed on the Vps24-Vps2 subcomplex as a membrane-bending and -buckling machine. Notably, either model may be dependent on Vps4, which could provide the energy for budding and scission through sequential or concerted removal of ESCRT-III subunits.

It is notable that these models are not completely mutually exclusive. Snf7 filaments may define a "zone of sequestration" for cargo that is further sculpted by the action of the Vps24-Vps2 subcomplex. This is supported by in vitro reconstitution of ESCRT-III assembly where membrane depressions were observed on liposomes when purified Vps20, Snf7, Vps24, and Vps2 were added in a ratio of ~1:10:5:3 but not seen when individual subunits were left out (Saksena et al., 2009). Both models also satisfy the ordered assembly of the ESCRT-III subunits.

Further study is needed to disentangle the true molecular mechanism of ILV generation. Because crystallography studies indicate that ESCRT-III subunits are structurally similar, how these small coiled-coil proteins achieve apparently diverse functions in the ESCRT pathway must also be investigated. Also, careful consideration must be given to how ESCRT-III mediates viral budding and cytokinesis. Because the topology of membrane deformation is conserved between these processes, it is reasonable to think that ESCRT-III functions in a similar capacity in each of them. Careful thought should also be given to the role of the other ESCRT complexes in vesicle formation. ESCRT-I forms an ~20 nm structure, and this raises the intriguing hypothesis that the elongated shape of ESCRT-I may play a dual role in both cargo binding and setting the size of an ESCRT-III ring. At the same time, the Y shape of ESCRT-II may govern the directions from which ESCRT-III filaments elongate to encircle cargo.

Closing

Diversity in ESCRT Function

Thus, from the perspectives of the ESCRT subunits, lipid membrane, and ubiquitinated cargo, the ESCRT pathway can be viewed primarily as a cargo-engaging and lipid bilayer-bending machine conserved from yeast to man. However, recent studies highlight an emerging divergence in ESCRT function. The most striking difference is the differential requirement of some ESCRT proteins in different biological contexts. As mentioned above, this is particularly apparent in retroviral budding. ESCRT-I and ESCRT-III subunits appear essential for viral release because Vps23/Tsg101 and Bro1/Alix bind directly to the L-domain of several viruses. However, ESCRTs -0 and -II and Vps20 appear dispensable for HIV-1 budding (Langelier et al., 2006). Similarly, ESCRT-I and -III are also important for cytokinesis, and their loss impairs abscission (Carlton and Martin-Serrano, 2007). Thus, ESCRT-0 appears negligible in both processes. This is reinforced by the observation that ESCRT-0 only appears present in animals and fungi, posing the question as to how the ESCRT pathway is initiated in other systems (Leung et al., 2008).

Bro1/Alix is an attractive candidate for Snf7 nucleation in processes that do not require ESCRT-II, but interestingly, Bro1 null yeast still contains assembled ESCRT-III complexes, suggesting that in yeast Bro1 is dispensable for Snf7 filament formation (Teis et al., 2008). Bro1 binds to the extreme C terminus of Snf7, allowing it to potentially modulate conformational changes in the C terminus of the protein.

Another significant divergence in ESCRT function is the difference in cargo between processes. MVB sorting requires ubiquitinated cargo, but ubiquitination of the "viral cargo" of an enveloped virus is not essential for its budding. ESCRTs do

not appear to utilize ubiquitin for cytokinesis either, where the "cargo" can be viewed as the entire daughter cell interior. This underscores the relative independence of each of the two chief ESCRT functions as a cargo-engaging and membrane-deforming machine.

Remaining Questions in the Field

In closing, since the discovery in 2001 and 2002 of the ESCRT complexes, amazing progress has been made toward the understanding of ESCRT function. Significant achievements have been made in ESCRT protein purification, molecular and genetic characterization, crystallization of intact ESCRT complexes, and reconstitution of ESCRT function in vitro. In parallel with this, the ESCRTs have emerged as key players in diverse cellular and viral processes. This has given insight into the potential ancestral purpose of the ESCRT proteins as membrane-deforming and scission machines necessary for cytokinesis in unicellular organisms—and it is notable that ESCRT-III and Vps4 are involved in cytokinesis in metazoans and *Crenarchaea*. ESCRTs -0, -I, and -II may represent a further specialization on this theme, coupling ubiquitin and PtdIns(3)P binding with scission to form a cargo-sorting pathway conserved from yeast to man. The ability to isolate and study distinct ESCRT complexes also underlines the "division of labor" between complexes necessary to carry out cargo sorting in a tightly regulated manner.

Despite all that we know, however, many questions still remain. In general, a complete elucidation of the ESCRT pathway will require a temporal and spatial understanding of: (1) cargo recognition and ordered recruitment of the ESCRT-0, -I, and -II complexes, (2) ordered assembly (i.e., Vps20, Snf7, Vps24, Vps2) at the membrane surface of ESCRT-III into a sequestering ring/spiral, (3) deubiquitination of cargo, (4) membrane deformation, (5) neck constriction and scission, and (6) Vps4-mediated ESCRT complex disassembly. At present, although stages one, two, and three are more clearly understood, stages four, five, and six require intense study to fully elucidate. To us, the most pressing is the mechanism of ESCRT-III-mediated scission. A deeper analysis of this will potentially aid in the understanding of viral budding, cytokinesis, and MVB sorting. Intertwined with this mystery is the need for an intact ESCRT-III three-dimensional structure, which will provide insight into its mechanism of action. Whether Vps4 helps to mediate scission or only disassemble ESCRT-III also remains a key question in the field.

Also needed is a greater understanding of the inter-ESCRT stoichiometry needed for a single round of MVB sorting. In vitro experiments on GUVs indicate that multiple ESCRT-0, -I, and -II complexes localize to forming ILVs, and further experiments are needed to confirm if this is true in vivo (Wollert et al., 2009). ESCRT-I and ESCRT-II form a supercomplex of 1:1 ratio in vitro (Gill et al., 2007). The ESCRT-0/ESCRT-I stoichiometry is less certain and may not be fixed for MVB sorting. Whether there is an exact stoichiometry to a budding event remains a major challenge to determine. Related to stoichiometry is the issue of how the ESCRTs spatially and temporally interact with ubiquitinated cargo at the endosome. Interestingly, recent studies have shown that mutating ubiquitin-binding moieties of ESCRT-I and -II separately do not result in sorting defects (Shields et al., 2009). This suggests that rather than function in a strictly processive series that can be interrupted if any one step is perturbed, the ESCRT complexes work synergistically to sort cargo.

There are also emerging differences between yeast and mammalian ESCRT pathways. Mammals contain multiple isoforms of ESCRT proteins, many with two or three paralogues. Whether these isoforms are tissue or pathway specific must be further elucidated. Intriguingly, overexpression of CHMP4a, b, and c isoforms elicits dominant-negative effects on viral budding and cytokinesis, but to very different degrees. CHMP4b most potently inhibits viral budding, but CHMP4c most potently inhibits cytokinesis (Carlton et al., 2008).

The role of lipids in ILV formation also remains a key question. Studies have indicated that the MVB sorting requires LBPA in higher eukaryotes, a lipid species that is not detected in yeast (Matsuo et al., 2004). *Crenarchaea* also have very different lipid compositions than mammals, yet both use ESCRTs for cell division. PtdIns(3)P is obviously crucial to the endosomal localization of the ESCRTs, but the impact of lipid composition for the budding and scission events requires further investigation.

The exact mechanism of ESCRT dysfunction in disease remains a key question in medical research. ESCRTs have been associated with numerous pathologies including cancer, AIDS, and neurodegeneration, and an understanding of exactly how perturbations in ESCRT function lead to disease will no doubt aid in the treatment of these diseases.

A final big question is whether additional processes involve ESCRT proteins. Autophagy and mitotic spindle maintenance have recently been implicated as ESCRT-dependent processes, but the mechanics of this need to be further elucidated (Lee et al., 2007; Morita et al., 2010).

In closing, the ESCRT field has made significant progress toward elucidating the mechanism-of-action of these distinctive protein complexes. Although they appear intricate, their function can be distilled down to the basic roles of cargo engagement and membrane deformation, which can be understood from the perspectives of the proteins and lipid bilayers involved. No doubt, future studies will continue to reveal exciting (and potentially unexpected) roles for the ESCRTs and Vps4 AAA ATPase.

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