The ESCRT machinery: From the plasma membrane to endosomes and back again

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Abstract
The manipulation and reorganization of lipid bilayers are required for diverse cellular processes, ranging from organelle biogenesis to cytokinetic abscission, and often involves transient membrane disruption. A set of membrane-associated proteins collectively known as the endosomal sorting complex required for transport (ESCRT) machinery has been implicated in membrane scission steps, which transform a single, continuous bilayer into two distinct bilayers, while simultaneously segregating cargo throughout the process. Components of the ESCRT pathway, which include 5 distinct protein complexes and an array of accessory factors, each serve discrete functions. This review focuses on the molecular mechanisms by which the ESCRT proteins facilitate cargo sequestration and membrane remodeling and highlights their unique roles in cellular homeostasis.

Introductory remarks
The collection of endosomal sorting complex required for transport (ESCRT) proteins consists of more than 30 gene products, many of which interact directly to generate distinct molecular machines that carry out a variety of cellular activities (Table 1). While the early-acting ESCRT complexes (ESCRT-0, ESCRT-I and ESCRT-II) assemble stably within the cytoplasm, the late-acting modules (ESCRT-III and Vps4-Vta1, which we refer to as ESCRT-IV) function specifically on membranes. A large proportion of these components have been characterized at angstrom resolution (reviewed extensively in (Hurley, 2010; Hurley & Emr, 2006; McCullough et al., 2013; Williams & Urbé, 2007), enabling a detailed understanding about how they associate with one another. Combined with genetic- and reconstitution-based studies, the assembly hierarchy of the ESCRT machinery on endosomal membranes has been determined, with ESCRT-0 acting most upstream to recruit ESCRT-I. The ESCRT-I complex engages ESCRT-II, which then nucleates ESCRT-III polymerization via the activation of the ESCRT-III subunit Vps20. ESCRT-IV ultimately disassembles the ESCRT-III complex to recycle its subunits. It is now clear that the ESCRT-III complex facilitates changes in membrane architecture, while the upstream components target ESCRT-III assembly to specific sites within cells. However, the field has yet to reach a consensus on the mechanisms by which the ESCRT machinery acts.

ESCRT-mediated membrane scission is an evolutionarily conserved process that is observed in disparate species, from archaea to eukaryotes. In all cases, ESCRT polymers assemble on the cytoplasmic surface of organelles to mediate membrane remodeling and ultimately facilitate topologically similar rearrangements. A series of genetic screens in yeast uncovered the majority of ESCRT subunits, all of which participate in the delivery of soluble and integral membrane hydrolytic enzymes to the lysosome lumen (Raymond et al., 1992; Rieder et al., 1996). Individual inhibition of these factors results in a distinctive phenotype that is highlighted by the formation of aberrant endosomes, consisting of numerous stacks of flattened cisternae, commonly known as class E compartments. While this effect is largely uniform in yeast, depletion studies in human cells reveal more variability, including the appearance of enlarged, swollen endosomes in some cases (Komada & Soriano, 1999; Raiborg et al., 2008). Irrespective of the morphological differences, the underlying mechanism governing the superfluous addition of membrane to endosomes may be shared. Sustained activity of the Rab5 GTPase promotes endosome fusion, resulting in organelles that have a similar surface area to those seen following ESCRT depletion in human cells (Stenmark et al., 2010). Moreover, function of the Rab5 isoform Vps21 is necessary to generate class E compartments in yeast cells that lack ESCRT components (Russell et al., 2012). These studies suggest that ESCRT dysfunction leads to an increase in Rab5 activity on endosomes, driving membrane accumulation and altering endosome morphology.
Table 1. Key components of the ESCRT machinery and their interacting partners.

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<td>PI3P, Ubiquitin, Tsg101, STAM, Clathrin</td>
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<td>Hrs</td>
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<tr>
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<td>Mvb12</td>
<td>PS, Tsg101, Vps37, Ubiquitin (yeast/UBAP1)</td>
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<td>Mvb12 A,B; UBAP1</td>
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<td>Vps22 (EAP30)</td>
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<td>ESCRT-III (Additional Factors)</td>
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<td>Vps4 A,B (SKD1)</td>
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<td>MIT, AAA, β-Domain</td>
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<td>Vps4, Vps60, Did2, Ist1, Vps20, Vps32, Vps24, Vps2</td>
<td>MIT, VSL</td>
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<td>Alix (AIP1)</td>
<td>Tsg101, Vps32, Cep55, DUBs</td>
<td>Bro1, PRR</td>
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Components of the ESCRT complexes and accessory factors are shown, with both metazoan and yeast nomenclatures provided. Also the important domains found in each factor and characterized binding partners for ESCRT components are listed. Please refer to the text for references. Abbreviations: Clathrin Binding (CB), Coiled-Coil (CC), C-Terminal Domain (CTD), Deubiquitinating Enzyme (DUB), Double-Sided Ubiquitin-Interacting Motif (DUIM), Fab-1, YGL023, Vps27, and EEA1 (FYVE), GRAM-Like Ubiquitin-binding in EAP45 (GLUE), Helical-Domain (HD), Mvb12-associated β-prism (MABP), Microtubule Interacting and Transport (MIT), N-Terminal Domain (NTD), Npl4 zinc finger (NZF), Phosphatidylinositol-3-Phosphate (PI3P), Proline Rich Region (PRR), Src homology-3 (SH3), Ubiquitin E2 variant (UEV), Ubiquitin Interacting Motif (UIM), Ubap1-Mvb12-Associated (UMA), Vps27p, Hrs, and STAM (VHS), Winged-Helix (WH).

Although a function for the ESCRT machinery was initially discovered in the endosomal system, its most ancient activity likely originates at the site of cell division (Samson et al., 2008). Homologues of several ESCRT-III and ESCRT-IV subunits have been identified throughout the crenarchaeal phylum, and those in Sulfolobus acidocaldarius were shown to direct the process of cytokinesis (Ellen et al., 2009; Lindás et al., 2008; Makarova et al., 2010). In an analogous manner, the ESCRT machinery plays important roles during cytokinetic abscission in animal cells and potentially plants (Carlton & Martin-Serrano, 2007; Morita et al., 2007b; Spitzer et al., 2006). In contrast, the yeast ESCRT complexes have not been found to participate directly in cytokinesis, but may instead contribute to the process of cell division through their roles in endocytic trafficking (McCullough et al., 2011). The absence of a specific role for the ESCRT machinery in yeast cytokinesis may be due to the lack of an appropriate adaptor protein. Mammalian cells use the microtubule bundling protein Cep55 to direct ESCRT components to the midbody, while archaea take advantage of an unrelated factor, CdvA (Carlton & Martin-Serrano, 2007; Dobro et al., 2013; Morita et al., 2007b; Samson et al., 2011). These findings suggest that the evolution of adaptors required for recruitment of ESCRT components to the cell division plane occurred independently in archaea and eukaryotes. Surprisingly, Cep55 homologues have not been identified outside of vertebrates, indicating that alternative mechanisms to enable ESCRT components to function in cytokinesis must exist in other metazoan organisms (Green et al., 2013). Additionally, non-host adaptors can also recruit the ESCRT machinery to the cell surface to support the budding of numerous enveloped viruses (reviewed extensively in McCullough et al., 2013; Meng & Lever, 2013; Votteler & Sundquist, 2013; Weissenhorn et al., 2013). Based on these data, it is reasonable to assume that other, yet to be identified adaptors exist, which can recruit ESCRT components to additional subcellular locations.

The goal of this review is to highlight recent developments that have shed light on our understanding of the mechanisms by which the ESCRT machinery acts. While we refer readers to other excellent reviews that have recently described the role of the ESCRT machinery in the budding of enveloped viruses (McCullough et al., 2013; Meng & Lever, 2013; Votteler & Sundquist, 2013; Weissenhorn et al., 2013), we will focus on the normal, physiological roles of ESCRT complexes in regulating the transport of membrane-associated proteins through the endocytic system and membrane abscission following cytokinesis. In particular, we will explore mechanisms that regulate the function and distribution of ESCRT components. It is now clear that the ESCRT machinery is not restricted to endosomes, as is suggested by its name.
Rather, a combination of protein and lipid interactions can direct ESCRT activity to multiple intracellular sites to facilitate membrane remodeling and budding reactions that sequester cargoes from the cytoplasm.

**ESCRT-mediated cargo sorting in the endolysosomal system**

Historically, the ESCRT system was first described as a network of complexes that function cooperatively to sort integral membrane proteins modified by ubiquitin (reviewed extensively in Hurley & Emr, 2006; Raiborg & Stenmark, 2009; Sakasena et al., 2007; Shields & Piper, 2011). The attachment of ubiquitin functions as a sorting signal to direct cargoes into intraluminal vesicles (ILVs) within endosomes, which ultimately fuse with lysosomes (Katzmann et al., 2004; Kölling & Hollenberg, 1994; Reggiori & Pelham, 2001). Incoming cargoes from the cell surface and the Golgi apparatus are among the best characterized substrates of this degradative pathway. In both cases, E3 ubiquitin ligases transfer ubiquitin onto one or more cytosol-facing lysine residues within substrates (reviewed extensively in Bonifacino & Traub, 2003; Hicke & Dunn, 2003; Hurley & Stenmark, 2011; Piper & Lehner, 2011). Although a single ubiquitin is sufficient for sorting into ILVs (Haglund et al., 2003; Stringer & Piper, 2011; Urbanowski & Piper, 2001), many cargoes undergo polyubiquitin-modification in vivo (Duncan et al., 2006; Geetha et al., 2005; Huang et al., 2006). In the case of epidermal growth factor receptor (EGFR), ligand stimulation results in a greater than 20-fold increase in ubiquitin modification relative to basal conditions (Huang et al., 2013). The majority of EGFR-associated ubiquitin is in the form of K63-linked chains (Argenzio et al., 2011; Huang et al., 2006, 2013; Meijer & van Leeuwen 2011), although K48-, K11- and K29-linked chains have also been observed (Huang et al., 2006). Dependencies on the presence of each chain type have yet to be determined for EGFR sorting. However, these data raise the possibility that an ubiquitin code acts to facilitate the segregation of EGFR when it transits through the endolysosomal system. Additionally, deubiquitinating enzymes (DUBs) and other ubiquitin ligases can further modify cargoes to specify their fate (Amerik et al., 2000; Ren et al., 2007; Shenoy & Lefkowitz, 2003). Thus, initial ubiquitin-modification does not necessarily target a protein for destruction, as subsequent editing can redirect its transport away from deposition into ILVs. A prime example of this phenomenon was demonstrated for AMSH, a DUB that interacts directly with components of the ESCRT machinery (Agromayor & Martin-Serrano, 2006; Kato et al., 2000; McCullough et al., 2004, 2006; Solomons et al., 2011; Tanaka et al., 1999). Depletion of AMSH, which alters the balance of E3 ligase/DUB activity in the endosomal system, enhances the degradation of EGFR (Bowers et al., 2006; McCullough et al., 2004), presumably by inhibiting the removal of ubiquitin from EGFR and thereby preventing its ability to recycle. However, the roles of DUBs in cargo sorting may not be so straightforward. Depletion of UBPY, another DUB that associates with ESCRT components (Kato et al., 2000; Row et al., 2007), has been shown to both accelerate and slow the rate of EGFR degradation (Bowers et al., 2006; Mizuno et al., 2005; Row et al., 2006). A basis for these contradictory findings remains unclear. However, one possibility is that DUBs not only target cargoes, but also components of the ESCRT machinery itself, which have been shown to be ubiquitin-modified by specific E3 ligases (e.g. Tal) in vivo (Amit et al., 2004; Jiao et al., 2009; Katz et al., 2002; Kim et al., 2007; Marchese et al., 2003). Attachment of ubiquitin to ESCRT subunits results in their inactivation (Hoeller et al., 2006; McDonald & Martin-Serrano, 2008), and DUB activity may therefore relieve inhibition of ESCRT-mediated sorting independently of ubiquitin removal from cargo. Alternatively, UBPY has also been proposed to disrupt binding of early-acting ESCRT components to HD-PTP, a member of the Bro1 family of accessory ESCRT proteins that regulates EGFR sorting at endosomes. By doing so, UBPY promotes the transfer of EGFR to late acting ESCRT complexes, while simultaneously facilitating the removal of ubiquitin from EGFR prior to its deposition into ILVs (Ali et al., 2013). This enables recycling of ubiquitin for future rounds of sorting. Thus, depending on the cell type, the interplay of DUB and ubiquitin ligase activities likely fine tune cargo sorting pathways, providing the high level of regulation necessary to control integral membrane protein homeostasis.

Members of the Cbl family of E3 ligases govern ubiquitin-modification of activated EGFR (Levkowitz et al., 1998, 1999; Waterman et al., 1999; Yokouchi et al., 1999). Although Cbl can associate directly with the ligand-bound receptor using a phosphotyrosine binding motif (Lill et al., 2000), it also interacts with Grb2, which acts as an EGFR-associated adaptor (Donovan et al., 1996; Meisner & Czech, 1995; Waterman et al., 2002). More generally, given the several thousand substrates that undergo lysosomal-mediated degradation, perhaps it is not surprising that a large family of adaptors for E3 ubiquitin ligases have evolved. For example, members of the Nedd4 family of E3 ligases have been shown to associate with beta-arrestin 2, which acts as a cargo-specific adaptor that enables ubiquitin modification of the beta2-adrenergic receptor following agonist stimulation (Han et al., 2013; Shenoy et al., 2008). Analogously, several arrestin-related trafficking adaptors (ARTs) have been discovered to function in the downregulation of specific cell surface proteins (Lin et al., 2008). Thus, the arrestins and ARTs represent a large group of adaptors that facilitate recognition of substrates by E3 ligases. Additionally, members of both families of adaptors associate directly with components of the ESCRT machinery, coupling E3 ligase activity to ESCRT function (Herrador et al., 2010; Malik & Marchese, 2010; Rauch & Martin-Serrano, 2011).

Early-acting complexes of the ESCRT machinery are ideally suited to recognize and sequester ubiquitin-modified cargoes (Bishop et al., 2002). Each harbors at least one ubiquitin-binding domain. However, based on interaction studies with soluble ubiquitin, the affinities for these domains are relatively weak, ranging from approximately 100-500 μM (Alam et al., 2004; Garrus et al., 2001; Mayers et al., 2011; Ren & Hurley, 2010; Slagsvold et al., 2005). Nevertheless, mutations that specifically inhibit ESCRT-mediated ubiquitin binding substantially impair cargo sorting to lysosomes, arguing a direct role for these domains in vivo.
expressed recombinantly, the proteins form an elongated 1:1 complex) and can engage two ubiquitin molecules simul-
taneously, as shown in Figure 1. The Hrs DUIM exhibits the tightest affinity for ubiquitin (\(K_m = 127 \mu M\)) based on isothermal titration calorimetry experiments using the intact complex) and can engage two ubiquitin molecules simul-
taneously (Hirano et al., 2006; Mayers et al., 2011). In a similar manner, the UIM and VHS domains of STAM can also associate with ubiquitin, albeit with weaker affinities (Ren & Hurley, 2010). In all cases, the interactions with mono-
ubiquitin are not cooperative (Mayers et al., 2011). However, the affinity of intact ESCRT-0 for tetraubiquitin (K63-linked) is approximately seven-fold higher, suggesting that cargoes modified with multiple ubiquitin molecules are likely to bind more avidly to ESCRT-0 through multiple simultaneous interactions (Ren & Hurley, 2010). It is important to note that in most cases, measured affinities underestimate the thermo-
dynamic parameters of ESCRT-0 associations with native cargoes. Since the substrate is integral to the membrane and not freely diffusible, equilibrium constants determined in solution must be converted to measurements in two dimen-
sions (Wu et al., 2011). Theoretically, based on a cis-acting association, this consideration would further increase the binding affinity of ESCRT-0 for ubiquitin, enabling cargo clustering, which is observed in vivo.

The architecture of the ESCRT-0 complex has been studied extensively. From cell extracts, Hrs and STAM co-purify in stoi-
chiometric quantities, suggesting that they associate constitutively (Ren et al., 2009). In addition, when co-
expressed recombinantly, the proteins form an elongated 1:1 heterodimeric complex (Mayers et al., 2011; Ren et al., 2009). The interface between the subunits has been resolved crystallographically and is composed of two domain-swapped GAT domains that are connected by an antiparallel coiled-coil (Prag et al., 2007; Ren et al., 2009). Based on molecular dynamics simulations, the intact ESCRT-0 complex exhibits a high degree of flexibility, which would enable its multiple ubiquitin-binding domains to lie in close proximity for cargo capture (Ren et al., 2009). Localization studies indicate that ESCRT-0 accumulates mostly on early endosomes. This distribution is directed, at least in part, by a FYVE domain within Hrs (Burd & Emr, 1998; Katzmann et al., 2003; Raiborg et al., 2001b), which exhibits nanomolar affinity for the endosomally-enriched phospholipid, phosphatidylinositol 3-phosphate (PI3P) (Stahelin et al., 2002). Mutations in the FYVE domain that abolish PI3P binding result in the cytosolic accumulation of ESCRT-0 (Katzmann et al., 2003; Raiborg et al., 2001b; Urbé et al., 2000). However, when expressed alone, the Hrs FYVE domain is insufficient to target to endosomes, suggesting additional requirements for ESCRT-0 membrane binding (Gillooly et al., 2000; Raiborg et al., 2001b).

Two crystal structures for the Hrs FYVE domain have been solved. While the yeast protein fragment is monomeric (Misra & Hurley, 1999), the structure obtained using Drosophila Hrs, which includes both the FYVE and the upstream VHS domains, reveals a potential for dimerization (Mao et al., 2000). In the latter structure, two FYVE domains are oriented in an antiparallel fashion and coordinated by citrate ions, which may mimic PI3P (Mao et al., 2000). When expressed in cells as a tandem dimer, the Hrs FYVE domain targets to endosomes efficiently, similar to the full-length protein (Hayakawa et al., 2004). These data suggest that the formation of an Hrs dimer may be a necessary prerequisite for its endosomal localization. Further support for the existence of an Hrs dimer in vivo comes from fluorescence resonance energy transfer (FRET)-based experiments, in which ectopically expressed CFP-Hrs undergoes unquenching upon photobleaching of YFP-Hrs on endosomes (Hayakawa et al., 2004), and from hydrodynamic studies conducted using Caenorhabditis elegans embryo extracts (Mayers et al., 2011). To resolve the apparent contradiction between cell-
based studies, which support the idea that Hrs exists as a dimer, and in vitro studies that argue that Hrs co-assembles with STAM in a 1:1 heterodimer in solution, recombinant ESCRT-0 was visualized on synthetic lipid bilayers using atomic force microscopy. These studies revealed that the Hrs:STAM heterodimer undergoes oligomerization specifically on membranes, generating mostly 2:2 heterotrimeric complexes (Mayers et al., 2011). The resulting complex brings together at least 8 low affinity ubiquitin-binding motifs, providing the avidity that is likely required for ubiquitin-dependent cargo sorting in vivo. Nonetheless, the minimal number of ubiqui-
tin-binding domains necessary for this process remains unclear, although it is likely that both Hrs and STAM contribute significantly.

The carboxyl-termini of the ESCRT-0 subunits largely function as interaction hubs. The SH3 domains of STAM associate with the DUBs, AMSH and UBPY, which were described earlier (Kato et al., 2000; Tanaka et al., 1999). Additionally, Hrs encodes a clathrin-interaction motif, enabling ESCRT-0 to associate with flat clathrin lattices that assemble on early endosomes (Raiborg et al., 2001a, 2002). These lattices likely facilitate clustering of ESCRT-0 into microdomains, which ultimately define sites of ILV formation. Deletion of the clathrin-binding box in Hrs results...
in a more uniform distribution on endosomes, consistent with its role in clustering ESCRT-0 (Raiborg et al., 2001a). However, the interaction between Hrs and clathrin may not be restricted to the endosome. Additional associations with several endocytic adapters, including the AP-2 and FCHO-Eps15-Intersectin (FEI) complexes, also target ESCRT-0 to clathrin-coated pits at the plasma membrane (Mayers et al., 2013). Although devoid of PI3P, the concentration of anionic phospholipids (e.g. phosphatidylserine) at the plasma membrane is relatively high (Leventis & Grinstein, 2010), which supports membrane association of ESCRT-0, in coordination with multiple protein-protein interactions. Notably, ESCRT-0 localizes to only a small subset of clathrin-coated pits, suggesting that additional regulatory mechanisms control its distribution on the cell surface (Mayers et al., 2013). One attractive possibility is that the concentration of ubiquitin-modified cargoes specifies the set of nascent clathrin-coated vesicles to which ESCRT-0 is recruited, thereby coupling ESCRT function to ubiquitin-mediated signaling during pit maturation (Henry et al., 2012).

Inhibition of ESCRT-0 recruitment to coated pits does not impair the rate of clathrin-mediated endocytosis, but instead slows the progress of ubiquitin-modified cargoes through the endolysosomal system (Mayers et al., 2013). These data support an early role for ESCRT-0 in substrate recognition at the plasma membrane, which facilitates rapid downstream sorting to lysosomes. There is currently no evidence to support an analogous role for ESCRT-0 at the Golgi. Instead, members of the GGA family of clathrin adaptors function in this capacity and engage biosynthetic cargoes through their low affinity (~180 μM), ubiquitin-binding GAT domains (Bilodeau et al., 2004; Puertollano & Bonifacino, 2004; Scott et al., 2004; Shiba et al., 2004). Each GAT domain is potentially capable of binding two ubiquitin molecules simultaneously, which may facilitate initial cargo recognition and concentration (Bilodeau et al., 2004; Prag et al., 2005). However, the mechanism underlying cargo transfer to the ESCRT machinery at the early endosome remains unclear.

In addition to its role in cargo sorting, ESCRT-0 also acts as a key adaptor for ESCRT-I recruitment to endosomes. Specifically, a PxxP motif within the Hrs carboxyl-terminus associates with a ubiquitin E2 variant (UEV) domain in Tsg101, one of the core ESCRT-I subunits (Bache et al., 2003; Bilodeau et al., 2003; Katzmann et al., 2003; Lu et al., 2003). However, the affinity of this interaction is weak (in the low μM range) and fails to support a constitutive localization of ESCRT-I to endosomal subdomains that contain ESCRT-0 (Ren & Hurley, 2011; Porillo et al., 2003). Nonetheless, depletion of ESCRT-0 inhibits ESCRT-I recruitment and function, indicating that a clear hierarchical assembly pathway exists for the ESCRT machinery in endosomal sorting (Bache et al., 2003; Katzmann et al., 2003). In species that lack ESCRT-0, including protists and plants, an alternative adaptor regulates ESCRT-I targeting to endosomes. The best candidates for this activity are members of the Tom1 family, which all harbor GAT domains that bind ubiquitin and additional motifs that associate with clathrin, Tsg101, and membrane phospholipids (Blanc et al., 2009; Herman et al., 2011; Shiba et al., 2004; Yamakami et al., 2003; Yanagida-Ishizaki et al., 2008). Tom1 and Tom1-like proteins are more highly conserved as compared to ESCRT-0, although their function(s) in animal cells have yet to be clearly defined (Seet et al., 2004).

The UEV domain of Tsg101 also binds to ubiquitin (Bilodeau et al., 2003; Katzmann et al., 2001; Porillo et al., 2002), albeit with poor affinity (~510 μM), utilizing a region that is distinct from the Hrs interaction motif (Garrus et al., 2001; Sundquist et al., 2004). These data suggest that the ESCRT-I complex may also participate in ubiquitin-dependent cargo sorting. However, a mutation in yeast Tsg101 that inhibits ubiquitin binding fails to affect the transport of several model ESCRT-dependent cargoes (Shields et al., 2009). Biochemical analysis of ESCRT-I has revealed a potential for numerous unique complexes to form in cells. In all cases, four distinct subunits (Tsg101, Vps28, a Vps37 isoform, and a Mvb12 or UBA1 isoform) co-assemble with a 1:1:1:1 stoichiometry to generate a heterotetramer in solution (Agromayor et al., 2012; Audhya et al., 2007; Kostelansky et al., 2007; Morita et al., 2007a) (Figure 2). Crystallographic and small-angle X-ray scattering (SAXS) studies focusing on yeast ESCRT-I indicate that the complex is elongated (~22.5 nm) with highly flexible termini that can engage ESCRT-0 and ESCRT-II, respectively (Boura et al., 2011;...
Kostelansky et al., 2007). However, a structure for metazoan ESCRT-I is lacking.

Although a specific function for human Mvb12 isoforms in endosomal protein sorting has yet to be demonstrated, UBAP1 and yeast Mvb12 have both been shown to bind ubiquitin and affect the kinetics of cargo degradation (Agromayor et al., 2012; Shields et al., 2009; Stefani et al., 2011). The affinity of yeast Mvb12 for ubiquitin has not been determined. However, structural studies place its ubiquitin-binding carboxyl-terminal near the UEV domain of Tsg101, suggesting both domains could bind with elevated avidity to a diubiquitin-modified cargo (Boura et al., 2011; Shields et al., 2009). Isothermal titration calorimetry studies indicate that UBAP1 associates with ubiquitin with low affinity (~140 μM in the context of an intact ESCRT-I complex), similar to the DUIM of Hrs (Agromayor et al., 2012). A solution structure of the UBAP1 carboxyl-terminal revealed the presence of overlapping ubiquitin associated (UBA) domains, which may engage up to three ubiquitin molecules simultaneously. However, binding studies indicate that the overlapping UBA domains bind equally to monoubiquitin as compared to diubiquitin (Agromayor et al., 2012).

In addition to its ability to associate with ESCRT-0, the ESCRT-I complex also associates weakly with acidic phospholipids, which are enriched on early endosomes (Boura & Hurley, 2012; Kostelansky et al., 2007). In particular, an amino-terminal basic patch in yeast Vps37, which is not conserved in higher eukaryotes (Kostelansky et al., 2007), and the MVB12-associated β-prism (MABP) domain of mammalian Mvb12 isoforms have been shown to bind phosphatidylserine-containing liposomes in vitro (Boura & Hurley, 2012). It is possible that additional domains of ESCRT-I also bind membrane phospholipids, but these may be difficult to identify, given the overall poor affinity of ESCRT-I for bilayers. Nonetheless, coincidence detection of ESCRT-0 and acidic phospholipids is likely responsible for directing ESCRT-I to associate transiently with endosomes in vivo.

Similar to ESCRT-I, the ESCRT-II complex does not stably associate with endosomes. However, interactions with acidic phospholipids and ESCRT-I enable its temporary recruitment (Teo et al., 2006). Biochemical studies indicate that ESCRT-II forms a stable heterotetramer in solution that is composed of 3 unique subunits: Vps22, Vps36, and two copies of Vps25 (Babst et al., 2002b; Im & Hurley, 2008) (Figure 3). Two of these bind to membranes, including the Gram-like ubiquitin-binding in Eap45 (GLUE) domain in Vps36, which exhibits a minor preference for PI3P and contributes little to ESCRT-II localization (Slagsvold et al., 2005; Teo et al., 2006), and a conserved, basic helical domain in Vps22 that nonspecifically associates with acidic phospholipids, but is required for ESCRT-II endosomal targeting (Im & Hurley, 2008). Although mammalian and yeast Vps36 differ in the organization of their GLUE domains, both can also bind to ubiquitin in a manner that is not competitive with phosphoinositides (Alam et al., 2004; Gill et al., 2007; Slagsvold et al., 2005). Additionally, the yeast GLUE domain contains two Npl4 type zinc fingers, the first of which binds to the carboxyl-terminal domain of the ESCRT-I subunit Vps28 (Gill et al., 2007), while the second binds to ubiquitin (Alam et al., 2004). Like other ubiquitin-binding domains identified in the early-acting ESCRT machinery, GLUE domains bind to ubiquitin with low affinity (~330 μM and ~182 μM for mammalian and yeast Vps36 isoforms, respectively) (Alam et al., 2004; Hirano et al., 2006; Slagsvold et al., 2005). It is important to note that irrespective of origin, ESCRT-II complexes contain only a single ubiquitin binding site. Some models suggest that cargo may be transferred from ESCRT-0 to ESCRT-I and/or ESCRT-II. However, mechanistic details to support this possibility have not been identified. Moreover, it is difficult to envision how ubiquitin-modified substrates would be released from ESCRT-0 in favor of binding to downstream ESCRT complexes, as both ESCRT-I and ESCRT-II harbor fewer ubiquitin-binding motifs (with similar or weaker affinities for ubiquitin) and neither stably associates with membranes. Although we cannot rule out the possibility of cargo transfer, a more likely scenario is that ESCRT-0 initially sequesters cargoes into endosomal microdomains, while simultaneously recruiting ESCRT-I and ESCRT-II to further inhibit lateral diffusion of substrates in the membrane. Dissociation of the early-acting complexes is likely coupled to DUB activity, but precisely how cargoes are efficiently deposited into ILVs remains a major outstanding question in the field. Although neither of the late-acting ESCRT complexes encodes cargo-binding motifs, ESCRT-II mediated assembly of ESCRT-III may locally retain cargoes within nascent vesicles until completion of the scission process (Teis et al., 2008; Wollert et al., 2009; Wollert & Hurley, 2010).

**ESCRT-mediated membrane bending**

In addition to sequestering ubiquitin-modified cargoes, the ESCRT machinery must also induce curvature at the
endosome limiting membrane to drive intraluminal vesicle formation. Both early- and late-acting components have been proposed to function in this capacity, and the field has yet to reach a consensus on the division of labor. Based on in vitro studies using giant unilamellar vesicles (GUVs) and purified yeast proteins, a combination of ESCRT-I and ESCRT-II were shown to generate inward budding structures that were several microns in diameter (Wollert & Hurley, 2010). However, for several reasons, the physiological relevance of these findings has been called into question. First, recombinant forms of yeast ESCRT-I and ESCRT-II bind avidly in solution with nanomolar affinity (Gill et al., 2007), a finding that contradicts studies using native forms of the complexes, which do not bind in yeast cell extracts (Babst et al., 2002b; Katzmann et al., 2001). Thus, key regulatory controls are absent in the studies that take advantage of these recombinant yeast complexes, complicating the interpretation of their membrane bending capabilities. Second, the binding interface between yeast ESCRT-I and ESCRT-II is not conserved in metazoan or plant systems, indicating that these complexes associate in a distinct (and yet to be fully defined) manner outside of fungi. Consistent with this idea, recombinant forms of C. elegans ESCRT-I and ESCRT-II do not bind with nanomolar affinity in solution nor on membranes, and incubation of these complexes with GUVs fails to induce bud formation above protein-free controls (our unpublished data). Additionally, incubation of human ESCRT-I and ESCRT-II with GUVs does not result in the formation of inward buds (Carlson & Hurley, 2012). Third, the size of nascent vesicles formed in the presence of recombinant yeast ESCRT-I and ESCRT-II (Wollert & Hurley, 2010) are two orders of magnitude larger than those expected, based on the average diameter of native ILVs (~25 nm in yeast) (Richter et al., 2007). A basis for this discrepancy is unclear, but could suggest that the membrane binding of the yeast ESCRT complexes stabilizes spontaneous deformations that occur on GUVs and promotes the formation of internal vesicles. Notably, the size of spontaneously formed vesicles within GUVs is similar to that observed upon addition of the yeast ESCRT machinery (Fyfe et al., 2011; Wollert et al., 2009).

An alternative model suggests a more direct role for the late-acting ESCRT machinery in generating membrane curvature on endosomes. In contrast to the early-acting complexes, ESCRT-III assembly occurs only on lipid bilayers in vivo (Babst et al., 2002a). The core ESCRT-III components, as defined in yeast, include Vps20, Vps32, Vps24 and Vps2, which are recruited sequentially (Teis et al., 2008). In addition, metazoan isoforms of Did2, Vps60, Ist1 and CHMP7 each play important, non-redundant roles in ESCRT-III function (Howard et al., 2001; Horii et al., 2006; Shim et al., 2006; Ward et al., 2005). The initial recruitment of Vps20 to endosomes is mediated by the Vps25 subunits of ESCRT-II, which exhibits a Y-shaped architecture based on crystallography studies (Hierro et al., 2004; Im & Hurley, 2008; Teo et al., 2004) (Figure 4). The two copies of Vps25 form distinct lobes that extend away from a third region that is formed by a heterodimer of Vps22 and Vps36. The ends of the Vps25 subunits in the complex possess exposed hydrophobic patches that bind directly to Vps20, likely in a conserved manner (Teo et al., 2004). Mutational analysis indicates that both copies of Vps25 are required for cargo sorting in vivo, suggesting that the nucleation of multiple ESCRT-III complexes by ESCRT-II is essential for function (Hierro et al., 2004; Teis et al., 2010).

All ESCRT-III proteins possess a similar domain organization, which includes a basic amino-terminus composed of two alpha helices and an acidic carboxyl-terminus harboring at least an additional three alpha helices. Fragments of three ESCRT-III subunits have been resolved by X-ray crystallography (Figure 5). These studies demonstrate that the basic helices of human Vps32 (hVps32), human Vps24 (hVps24), and Ist1 exhibit extensive similarity, forming a ~7 nm helical hairpin (Bajorek et al., 2009; Lata et al., 2008a; Martinelli et al., 2012; Muziol et al., 2006). Additionally, the structure of hVps24 derived from residues 9–183 revealed that the adjacent helices (α3 and α4) pack against the open end of the helical hairpin asymmetrically to generate a four-helix bundle (Muziol et al., 2006). The fifth helix (α5) is connected to the rest of hVps24 by a disordered linker of ~20 amino acids. The analogous region of Ist1 (residues 1–189) exhibits a

Figure 4. ESCRT-II binds to Vps20 to initiate ESCRT-III polymerization. Vps20 is believed to exhibit a closed conformation within the cytoplasm and is unable to interact with ESCRT-II. However, upon recruitment to the endosomal membrane by ESCRT-II, Vps20 autoinhibition is relieved by a mechanism that is currently undefined. (see colour version of this figure at www.informahealthcare.com/bmg).
similar architecture with respect to the 4-helix bundle (Bajorek et al., 2009). However, one striking difference between the hVps24 and Ist1 structures is the position of helix 5. In Ist1, this acidic helix packs against the closed end of the hairpin, resulting in a “closed” conformation (Bajorek et al., 2009), while the analogous helix in hVps24 fails to contact the helical hairpin, generating an “open” conformation (Muzioł et al., 2006). Based on these structures and prediction models, all ESCRT-III subunits are likely to adopt the 4-helix bundle configuration, with a fifth helix connected by a highly flexible linker. In addition, secondary structure prediction algorithms propose the presence of a sixth helix downstream of z5 in most cases (Shim et al., 2007). However, this domain has only been observed in a single Ist1 crystal form, under conditions where it is stabilized by lattice contacts (Bajorek et al., 2009). In the context of other ESCRT-III subunits, the dynamic flexibility of z6 likely prevents its visualization by X-ray crystallography.

Crystallization of hVps24 results in multiple structures with distinct interfaces (Bajorek et al., 2009; Muzioł et al., 2006). Although it remains unclear whether any are physiologically relevant, a “tip-to-tip” orientation in which the helical hairpin loops of two monomers associate, has been observed in several crystal forms. This interaction is only feasible if helix 5 is displaced away from the helical hairpin, and together with the Ist1 crystal structure, supports the idea that ESCRT-III monomers can adopt multiple conformations that either promote or restrict dimerization. The closed conformation appears to correlate with an autoinhibited state, in which helix 5 blocks interactions with other ESCRT-III subunits (Bajorek et al., 2009; Lata et al., 2008a). By disrupting this intramolecular interaction, which likely results in the transition to an open conformation, several studies have demonstrated that ESCRT-III subunits become activated and exhibit the tendency to polymerize in an unregulated manner (Bajorek et al., 2009; Henne et al., 2012; Lata et al., 2008b; Muzioł et al., 2006; Teis et al., 2008; Zamborlini et al., 2006). When expressed in cells, these activated forms of ESCRT-III impair the function of the ESCRT machinery, due to their ability to oligomerize even in the absence of upstream stimulation (Bajorek et al., 2009; Dukes et al., 2008; Shim et al., 2007; Zamborlini et al., 2006). Thus, the open

Figure 5. Overlays of solved ESCRT-III structures. (Top) Representative image from the overlay of the helical hairpin region for human Vps32B (PDB code: 4ABM) and human Vps24 (3FRT). (Middle) Representative image from the overlay of the structures for human Vps24 in the proposed closed (PDB code: 3FRT) and open (PDB code: 2GD5) conformations. (Bottom) Representative image from the overlay of the structures for human Vps24 (PDB code: 3FRT) and human Ist1 (3FRR). (see colour version of this figure at www.informahealthcare.com/bmg).
conformation observed in the monomeric hVps24 crystal structure likely represents its activated state.

Numerous ESCRT-III proteins have been shown to polymerize, either on their own or together with another subunit, to generate structures of various sizes and shapes (Bodon et al., 2011; Ghazi-Tabatabai et al., 2008; Hanson et al., 2008; Henne et al., 2012; Lata et al., 2008b; Pires et al., 2009). Wild-type yeast Vps32 exhibits a high degree of heterogeneity, forming sheets, rings and filaments (Ghazi-Tabatabai et al., 2008). However, when mutated to relieve autoinhibition and placed on a lipid monolayer, spiral filaments are observed with a thickness of ~9 nm (Henne et al., 2012). Further analysis revealed that each filament was composed of two intertwined subfilaments. The physiological relevance of these protofilaments is unclear, as their formation requires relatively high concentrations of purified protein (at least 12.5 μM) (Henne et al., 2012). Additionally, overexpressed human Vps32 forms similar spiral shaped filaments at the cortex of tissue culture cells, but they exhibit a thickness of only ~5 nm (Hanson et al., 2008). Which of these structures is representative of the native Vps32 polymer remains to be shown.

In contrast to the structures assembled by Vps32, yeast Vps24 forms helical filaments with a diameter of ~15–20 nm when purified at high concentrations (~380 μM) (Ghazi-Tabatabai et al., 2008). Similarly, activated forms of human Vps24 and Vps2, generated by truncating their carboxytermini, co-assemble to generate ~40 nm wide helical tubes (Lata et al., 2008b). When combined with liposomes, these truncated proteins drive the formation of ~50–100 nm wide tubules that narrow and close to form a dome. Similarly, overexpression of human Vps2 drives the formation of tubular protrusions that extend away the cell surface and are often shed into the media (Bodon et al., 2011). These membrane-coated tubes exhibit variability in their diameter (~70–350 nm), but numerous constrictions are observed (to ~16 nm in diameter) and the ends of tubes close with a dome-like architecture. Both Ist1 and hDid2 also homo-polymerize to generate helical tubes that are ~700 nm and ~230 nm in diameter, respectively (Bajorek et al., 2009). These ESCRT-III proteins have been shown to interact directly and can co-assemble to generate similar helical assemblies. Collectively, these findings demonstrate a clear distinction between the types of polymers generated by Vps32 and the subsequent acting ESCRT-III subunits (Vps24, Vps2, hDid2 and Ist1), suggesting these factors act at unique steps of ESCRT-mediated membrane remodeling.

As described earlier, a complex composed of ESCRT-II and Vps20 initiates polymerization of endosomal ESCRT-III. Biochemical studies using purified yeast proteins suggest that this nucleating supercomplex forms only on lipid bilayers (Henne et al., 2012; Teis et al., 2010). Moreover, ESCRT-II binding promotes a conformational change in Vps20, as measured indirectly using environmentally sensitive dyes, suggesting that it relieves Vps20 autoinhibition and fosters its association with Vps32 (Sakseka et al., 2009). An X-ray structure of the ESCRT-II:Vps20 interface has been solved, showing that the binding region on Vps20 is restricted to a region of α1 that is distal to the helical hairpin loop that is predicted to contact α5 in the autoinhibited state (Im et al., 2009). Based on these findings, it is unclear why the ESCRT-II:Vps20 interaction is restricted to membranes. Moreover, a mechanism underlying the proposed activation of Vps20 by ESCRT-II is difficult to fathom, based on their mode of interaction. Due to these apparent incongruities, an alternative interpretation of the spectroscopic data generated using purified proteins and liposomes may be required. It is important to note that a combination of ESCRT-II and Vps20 binds to liposomes with considerably tighter affinity, as compared to either component alone (Fyfe et al., 2011; Im et al., 2010; Teo et al., 2004). Thus, changes in the spectral properties of Vps20 upon ESCRT-II binding may be a result of this effect, as opposed to a relief in autoinhibition.

In addition to the improved membrane binding affinity exhibited by ESCRT-II:Vps20, the supercomplex exhibits a preference for membranes of elevated curvature (Fyfe et al., 2011). Furthermore, based on liposome binding experiments, the ESCRT-II:Vps20 complex behaves in a mechanosensitive manner, responding to increases in membrane curvature by binding more tightly (Fyfe et al., 2011). These studies raise the possibility that ESCRT-II and Vps20 induce membrane curvature at endosomes. In this scenario, an initial deformation of the limiting membrane, potentially generated through ESCRT-mediated clustering of ubiquitin-modified transmembrane proteins (McMahon & Gallop, 2005), would specify a microdomain to which the ESCRT-II:Vps20 complex would be targeted. Binding to a region of elevated curvature may lead to additional membrane deformation, further increasing the curvature at this site. Ultimately, through this feedforward mechanism, a nascent vesicle could form, but ESCRT-II:Vps20 would be restricted to the curved bud neck. Consistent with this idea, a structural model of the ESCRT-II:Vps20 supercomplex has been suggested to promote negative membrane curvature and docks onto a concave membrane with a radius of curvature similar to that observed in metazoan ILVs (Im et al., 2009). Nevertheless, validation of such a model requires additional testing.

Yet another possibility is that downstream ESCRT-III components coordinately drive membrane invagination to create vesicles that bud away from the cytoplasm. Several studies have shown that Vps32 forms flat spirals and circular arrays on lipid bilayers, both in vitro and in vivo, which are confined mostly to a single plane (Hanson et al., 2008; Henne et al., 2012). However, these structures can be dramatically remodeled to create three-dimensional coiled helices upon the addition of downstream ESCRT-III subunits (Vps24 and Vps2) or a mutant form of the AAA ATPase Vps4, which cannot hydrolyze ATP (Hanson et al., 2008; Henne et al., 2012). The diameter of the helical tubes that appear when Vps24 and Vps2 are added to pre-existing Vps32 flat spirals is distinct from that observed using truncated forms of Vps24 and Vps2 (~85 nm versus ~40 nm, respectively) (Henne et al., 2012; Lata et al., 2008b). These data support the idea that Vps32 spiral filaments transition from a planar arrangement to a more extended conformation, as opposed to nucleating Vps24:Vps2 helices. Such reorganization in Vps32 filaments could result in membrane deformation, although it is unclear how a spherical ILV, shown to be ~50 nm in diameter in metazoan cells and devoid of internal ESCRT-III, would be generated in this scenario. Based on the
analysis of flat Vps32 spirals that are remodeled in cells through an association with an ATPase deficient form of Vps4, 100–120 nm buds and helical tubes that are scaffolded by ESCRT-III filaments are observed (Hanson et al., 2008). As ESCRT components are not consumed during the process of ILV budding, these structures are unlikely to be related to intermediates in the vesicle budding process in vivo. For an analogous reason, membrane deformations generated by Vps24:Vps2 helical tubes also cannot explain the initiation of vesicle formation at endosomes.

ESCRT-mediated membrane scission

The process of vesicle biogenesis concludes with a membrane scission step that results in a complete separation of the donor membrane from the vesicle. There is a wide consensus in the field that the ESCRT-III complex is principally involved in the release of vesicles into the endosome lumen, but the mechanism remains undefined. Deletion of any core ESCRT-III subunit in yeast blocks ILV formation (Babst et al., 2002a). Additionally, using a GUV-based assay, recombinant forms of Vps20, Vps32 and Vps24 have been shown to be sufficient to form large, nascent buds and release them into the GUV lumen, although high concentrations of each factor were necessary to mediate both steps (Wollert et al., 2009). Surprisingly, these studies also indicated that Vps2 and ESCRT-IV were dispensable for membrane scission, suggesting that their major contribution was to recycle ESCRT-III components off the membrane subsequent to their action. An alternative viewpoint argues for a more active role for ESCRT-IV in vesicle release, through its ability to bind, restructure and disassemble ESCRT-III filaments (Hanson et al., 2008; Henne et al., 2012; Saksgna et al., 2009). Considering that ESCRT-IV binds to all ESCRT-III subunits, albeit with varying affinities (Azmi et al., 2008; Kieffer et al., 2008; Merrill & Hanson, 2010; Shim et al., 2008; Skalicky et al., 2012; Stuchell-Breerton et al., 2007; Xiao et al., 2008), and encodes the only known enzyme that functions in the absence of nucleotide, Vps4 exists in an equilibrium between monomer and dimer states, neither of which exhibits activity (Babst et al., 1998; Gonciarz et al., 2008; Hartmann et al., 2008; Xiao et al., 2007, 2008). However, upon ATP binding, Vps4 oligomerizes to an active form. Mutating a conserved Walker B glutamate residue to glutamine allows for ATP binding and Vps4 oligomerization, but prevents ATP hydrolysis and complex disassembly (Azmi et al., 2006; Babst et al., 1997; Scott et al., 2005a; Stuchell-Breerton et al., 2007; Whiteheart et al., 1994; Xiao et al., 2007, 2008). Using this mutant isoform, studies have shown that yeast Vps4 assembles into dodecameric or tetradecameric complexes that resemble pairs of stacked rings and share the feature of a central pore, which is required for function (Hartmann et al., 2008; Landsberg et al., 2009; Yu et al., 2008). Models suggest that ESCRT-III subunits contact the pore directly and potentially pass through the stacked ring structure, leading to disassembly of filaments. However, direct evidence in support of these models is lacking. Moreover, recent work examining the oligomeric state of wild-type yeast Vps4 in the presence of ATP contradicts prior studies and indicates that Vps4 self-associates to form mostly hexamers in a concentration-dependent manner, a finding that is consistent with the assembly mode of other Type I AAA ATPases (Monroe et al., 2013; Roll-Mecak & Vale, 2008; Zhang et al., 2000). These data argue that the stacked ring structures that assemble using mutant, ATPase-deficient Vps4 may not be physiologically relevant. Consistent with this idea, archaeal Vps4 also forms hexamers, but typically not higher order oligomers, in the presence of ATP (Monroe et al., 2013). Thus, the mechanism by which Vps4 acts on ESCRT-III complexes may be more similar to that of other Type I AAA ATPases (e.g. spastin) than previously thought.

Vta1 binds directly to Vps4 and has been suggested to potentiate its activity (Azmi et al., 2008; Norgan et al., 2013; Shim et al., 2008) and stabilize its oligomeric conformation (Azmi et al., 2006). This regulatory subunit of ESCRT-IV contains two amino-terminal MIT domains that associate with a subset of ESCRT-III subunits with low micromolar affinity and a carboxyl-terminal VSL domain, which binds to the β-domain of Vps4 (Agromayor et al., 2009; Scott et al., 2005b; Shim et al., 2008; Skalicky et al., 2012; Yang & Hurley, 2010). In isolation, Vta1 forms stable dimers that were suggested to stabilize the dodecameric architecture of ATPase-deficient Vps4 (Azmi et al., 2006). However, more recent data indicate that Vta1 does not alter the stoichiometry of the more native Vps4 hexamer in the presence of ATP (Monroe et al., 2013). Although this finding does not contradict prior work demonstrating that Vta1 stimulates Vps4 ATPase activity, its regulatory mechanism must be significantly more complex than is currently appreciated. Deletion of Vta1 in yeast inhibits the normal trafficking of ubiquitin-modified cargoes in the endolysosomal system, but the effect is not nearly as severe as loss of other ESCRT components (Azmi et al., 2006; Rue et al., 2008; Shiflett et al., 2004). Similarly, depletion of LIP5 impairs receptor downregulation in mammalian cells (Ward et al., 2005). These studies indicate that Vta1-mediated stimulation of Vps4 is important for the kinetics of cargo transport to the lumen of endosomes, potentially by accelerating the rate of
ESCRT-III complex disassembly during membrane scission events. In addition, the Vta1 MIT domains can further direct ESCRT-IV assembly to ESCRT-III containing membrane microdomains.

Vps4 activity is further regulated by its substrate, ESCRT-III. The amino-terminal MIT domain, which forms a three-helix bundle, can independently engage two distinct motifs in ESCRT-III subunits known as MIM1 and MIM2 (Kieffer et al., 2008; Obita et al., 2007; Stuchell-Brereton et al., 2007). Helices 2 and 3 mediate the MIM1 interaction, while helices 1 and 3 form a unique binding pocket to associate with MIM2. Vps24, Vps2 and Did2 harbor only MIM1 motifs, while Vps20 and Vps32 harbor only MIM2 domains in their acidic carboxyl-termini. In contrast, Ist1 and CHMP7 contain both motifs. While yeast Vps4 is stimulated by MIM1 motifs only, all MIM domains (with the addition of ~50 residues of upstream sequence) activate mammalian Vps4, highlighting a key distinction between the yeast and metazoan ATPases (Azmi et al., 2008; Merrill & Hanson, 2010). In vitro, metazoan Vps4 exhibits extremely little activity in the absence of ESCRT-III subunits, indicating that substrate recognition acts as a key spatial and temporal regulator of ESCRT-IV function (Merrill & Hanson, 2010). Deletion of the Vps4 MIT domain and an adjacent linker increases its basal activity, suggesting that this region normally acts in an autoinhibitory fashion (Babst et al., 1998; Merrill & Hanson, 2010). Relief of autoinhibition through interactions with ESCRT-III subunits likely plays a key role in activating Vps4. Additionally, the acidic nature of the ESCRT-III carboxyl-termini appears to be essential for this function, as other highly acidic molecules (e.g., poly-L-glutamic acid) can also increase Vps4 activity independently of ESCRT-III (Merrill & Hanson, 2010). Together, these findings support a model in which MIT–MIM interactions facilitate the positioning of acidic residues near the ATPase domain to alter its rate of ATP hydrolysis, which is necessary to dismantle ESCRT-III polymers. In an analogous manner, the carboxyl-terminal acidic residues in α-tubulin are critical in activating the AAA ATPase spastin, which severs polymerized microtubules (Roll-Mecak & Vale, 2008; White et al., 2007).

To couple ESCRT-III disassembly to membrane scission, the action of ESCRT-IV must be tightly regulated. In this model, the stepwise removal of ESCRT-III subunits from a pre-existing polymer would tighten the circumference of the vesicle bud neck and facilitate closure. Several prerequisites must be satisfied to consider this hypothesis further. First, ESCRT-III should be anchored to the highly curved membrane at the neck of the nascent vesicle. As described previously, a complex of ESCRT-II and Vps20 displays curvature sensitivity, binding more avidly to highly bent lipid bilayers (Fyfe et al., 2011). Moreover, visualization of ESCRT-II:Vps20-nucleated polymers of Vps32 on bilayers with varying degrees of curvature demonstrated that they too exhibit a strong preference for curved membranes (Fyfe et al., 2011). Additionally, Vps24, Vps2, Did2 and Ist1 are all capable of assembling to helical complexes that deform membranes into tubes of high curvature (Bajorek et al., 2009; Bodon et al., 2011; Lata et al., 2008b). A basis for ESCRT-III membrane binding comes from their three-dimensional structures (solved or modeled), which exhibit electrostatic patches in their common asymmetric 4-helix bundles (Buchkovich et al., 2013). These basic regions, largely found in helices 1, 2 and/or 3, are conducive to binding anionic phospholipids, which are enriched on endosomal membranes. Vps20 is also predicted to undergo myristoylation near its amino-terminus, which would further foster its membrane association (Babst et al., 2002a). More recently, several ESCRT-III subunits have been proposed to contain an amino-terminal motif that can insert into membranes (Buchkovich et al., 2013). Although their depths of penetration have yet to be characterized, spectroscopic measurements suggest that yeast Vps32 can delve partially into bilayers, which would induce membrane curvature. These findings collectively support the idea that ESCRT-III adheres tightly to curved membranes.

Second, ESCRT-IV activity should result in constriction of ESCRT-III polymers as they disassemble to bring opposing membranes in the vesicle bud neck closer together. Relatively few studies have directly examined ESCRT-III remodeling in the presence of active ESCRT-IV. Electron microscopy-based studies indicate that Vps4 binds to the inside of helical tubes composed Vps24 and Vps2 in the absence of ATP and drives their disassembly when ATP is added (Lata et al., 2008a). However, these findings did not clarify the mechanism by which Vps4 alters the helical organization of Vps24-Vps2 polymers that results in disassociation. In cells, Vps32 filaments that assemble upon overexpression bind to an ATPase-deficient form of Vps4 along their entire length (Hanson et al., 2008). In this case, Vps4 binding results in the compaction of Vps32 circular arrays, which is consistent with an active role for ESCRT-IV in reorganizing ESCRT-III architecture, but uncouples the disassembly reaction from constriction. Together, these findings suggest that the current model for ESCRT-IV driven membrane scission requires revision. Instead of acting in a single reaction, ESCRT-IV may function in multiple steps. In an initial binding reaction, ESCRT-III locally concentrates ESCRT-IV, driving its oligomerization into hexameric ring structures. Coupled to this process, ESCRT-IV promotes the constriction of ESCRT-III spiral filaments. In this manner, the opposing membranes in a vesicle bud neck could be brought into close proximity. Finally, interactions between ESCRT-III MIM domains and Vps4 MIT domains allow the acidic motifs in ESCRT-III subunits to activate the Vps4 ATPase, driving disassembly of ESCRT-III polymers, and simultaneously releasing energy to facilitate membrane scission.

An alternative to this model is a more direct role for Vps24:Vps2 in promoting membrane constriction. Based on the architecture of the ends of helical tubes generated by recombinant Vps24 and Vps2, several in the field speculate the these dome-like structures promote membrane sealing during ILV budding (Lata et al., 2008b). Indeed, assembly of such a dome at the vesicle bud neck could draw opposing membranes together to facilitate scission (Elia et al., 2012; Fabrikant et al., 2009). Additionally, based on the hierarchy of ESCRT-III filament assembly, Vps24 and Vps2 are recruited by Vps32, allowing them to act late in the vesicle formation process (Teis et al., 2008). With such a role as is proposed for Vps24:Vps2, ESCRT-IV activity becomes less relevant to scission, although it continues to be required for ESCRT-III
disassembly and subunit recycling. Detection of dome-like structures composed of Vps24 and Vps2 have yet to be identified in a native setting, raising concerns about this model. However, their existence may be extremely transient, impeding progress toward their visualization.

It is also feasible that the mechanism of membrane scission requires elements from multiple models (Figure 6). As the initial curvature sensor in the ESCRT machinery, a complex of ESCRT-II and Vps20 may serve to induce membrane bending and vesicle formation at sites where ESCRT-0 has clustered ubiquitin-modified cargoes. Although the mechanism by which Vps32 polymerization is triggered by ESCRT-II:Vps20 remains unclear, it may require a specific conformation of Vps20 that is obtained when the membrane is appropriately bent. Consistent with this idea, ESCRT-II:Vps20 can nucleate Vps32 polymers on areas of high curvature, but not on flat membranes (Fyne et al., 2011). Cargo diffusion would be restricted by the formation of Vps32 spiral filaments, potentially in a manner akin to that of septin filaments, which create a membrane diffusion barrier between mother and daughter cells in yeast (Hu et al., 2010). Additionally, Vps32 has been shown to recruit DUB enzymes in yeast, which would recycle ubiquitin present on cargo molecules (Ali et al., 2013). The mechanism by which Vps24 binds to Vps32 polymers is unclear. However, their association results in remodeling of Vps32 spiral filaments, which may cause their further penetration into the bud neck (Henne et al., 2012). Additionally, Vps24 has been shown to recruit AMSH in mammalian cells, further supporting cargo deubiquitination (Agromayor & Martin-Serrano, 2006; Kiyuma et al., 2007; Ma et al., 2007; McCullough et al., 2006; Solomons et al., 2011; Tsang et al., 2001; Zamborlini et al., 2006). Formation of a Vps24:Vps20 dome-like structure within the neck would reorganize the membranes, bringing opposing bilayers together within the neck. The final action of ESCRT-IV would further restructure spiral helices within the neck and facilitate spontaneous scission, but only when the membranes there are properly juxtaposed. Further contributions to this set of reactions by the other ESCRT-III components (Ist1, Did2 and Vps60), all of which can modulate Vps4 ATPase activity (Azmi et al., 2008; Dimaano et al., 2008; Rue et al., 2008; Merrill & Hanson, 2010; Nickerson et al., 2010), would promote scission and subsequent recycling of ESCRT-III subunits after membrane resealing. In particular, the stepwise co-assembly of ESCRT-III subunits would progressively generate a high avidity binding site for ESCRT-IV, both temporally and spatially regulating its action.

**ESCRT function during cytokinetic abscission**

Cytokinesis is the process that divides two daughter cells from one another after the completion of mitosis. In animal cells, an actin-myosin ring constricts the plasma membrane to create a thin (~200 nm) intracellular bridge, which must ultimately be resolved by a membrane scission step (Adams et al., 1998; Green et al., 2013; Madaule et al., 1998). Within this structure resides the midbody, which assembles from tightly packed antiparallel microtubules that initially contributed to chromosome segregation (Hu et al., 2012; Mullins & McIntosh, 1982; Saxton & McIntosh, 1987), and the midbody ring, which consists of contractile ring components, including actin and myosin (Breckler & Burnside, 1994; Pohl & Jentsch, 2008; Sanger et al., 1989). In contrast to ILV formation at endosomes, the requirement for the ESCRT machinery to induce membrane curvature is bypassed during abscission. By comparing the set of ESCRT components required for these processes, several conspicuous differences are elucidated. First, ESCRT-0 does not function during cytokinasis, as cargo clustering is not required. However, since ESCRT-I associates weakly and nonspecifically with membranes, a distinct adaptor must exist to localize it and downstream components of the ESCRT machinery appropriately. In mammalian cells, the microtubule bundling protein Cep55 has been shown to bind directly to the Tsg101 subunit of ESCRT-I, enabling it to accumulate at the midbody (Carlton & Martin-Serrano, 2007; Morita et al., 2007b). However, Cep55 is vertebrate-specific, indicating that another factor must exist to mediate ESCRT-I recruitment to the midbody in other metazoans. Consistent with this idea, studies in *C. elegans* have demonstrated that ESCRT-I accumulates at midbodies during embryo development (Green et al., 2013), even though the GPPX3Y motif in human Tsg101 that binds to Cep55 (Lee et al., 2008) is absent in *C. elegans* Tsg101. Additionally, depletion studies show that a small percentage of mammalian cells depleted of Cep55 (~10–30%) can continue to undergo cell division (Carlton & Martin-Serrano, 2007; Morita et al., 2007b). Thus, an alternative mechanism to recruit ESCRT-I to the midbody exists and may be functionally conserved in all eukaryotes.

Although ESCRT-I participates in both ILV formation and abscission, depletion of its binding partner ESCRT-II does not block cell division (Langelier et al., 2006; Malerød et al., 2007). Additionally, localization studies have failed to show that components of ESCRT-II accumulate at the midbody during cytokinesis. One potential reason for its absence at the midbody is the low levels of PI3P at this site, which normally plays a role in ESCRT-II membrane association on endosomes (Gillooly et al., 2000; Slagsvold et al., 2005). Additionally, depletion of the Vps20 subunit of ESCRT-III
results in very few multinucleated cells, as compared to the depletion of other ESCRT-III activities (Morita et al., 2010). Notably, unlike most ESCRT-III subunits in human cells, Vps20 function is contributed by a single isoform. Thus, if its functions were important during abscission, as appears to be the case for all other ESCRT-III components (Morita et al., 2010), one would anticipate a significant increase in cell division defects upon its loss. Also, without its upstream binding partner ESCRT-II, it is unclear how Vps20 would localize to the midbody, especially given its weak affinity for membranes in isolation (Fyle et al., 2011; Teo et al., 2004). Perhaps it is not surprising that ESCRT-II and Vps20 are not required in abscission, as their major role in ILV formation may rely mostly on their ability to induce the initial stages of membrane bending, a function that is unnecessary in the context of actin-myosin driven abscission.

In contrast to the dispensable functions of ESCRT-0, ESCRT-II and Vps20 in cytokinesis, some ESCRT components appear to have more significant roles in this process as compared to ILV biogenesis. In particular, depletion of the late-acting ESCRT-III subunits Ist1 and Did2 results in a substantial increase in multinucleated cells, indicative of a defect in cytokinesis (Agromayor et al., 2009; Bajorek et al., 2009; Morita et al., 2010). However, mutations in these components were not isolated in the original genetic screens that identified the majority of ESCRT components in yeast (Raymond et al., 1992). Moreover, deletion of Ist1 or Did2 individually does not cause a class “E” phenotype, indicating a more limited role in ILV biogenesis (Rue et al., 2008). These data suggest that the helical polymers generated by Ist1 and Did2 may function in a more abscission-centric manner, potentially facilitating recruitment of ESCRT-IV in a manner that is not required during ILV scission. Similarly, deletion of Alix, an accessory component of the ESCRT machinery, results in a very weak class “E” phenotype in yeast, but its inhibition in human cells causes a dramatic defect in cytokinesis (Carlton & Martin-Serrano, 2007; Carlton et al., 2008; Morita et al., 2007b; Raymond et al., 1992). Alix binds to both Tsg101 (Martin-Serrano et al., 2003; von Schwedler et al., 2003) and Vps32 (McCullough et al., 2008; Pires et al., 2009), raising the possibility that it bridges ESCRT-I and ESCRT-III and enables bypass of ESCRT-II during midbody resolution. However, in vitro reconstitution experiments have failed to demonstrate that ESCRT-I and Alix can promote assembly of ESCRT-III polymers (Carlson & Hurley, 2012). Thus, it remains unclear how ESCRT-III filament assembly is initiated within intracellular bridges.

Another key difference between ILV formation and abscission is the timescale of the reactions. While ILVs are generated on the order of seconds to minutes, resolution of the midbody in mammalian tissue culture cells requires ~1–3 hours or more (reviewed extensively in Barr & Gruneberg, 2007; Eggert et al., 2006; Steigemann & Gerlich, 2009). The dynamics of the ESCRT machinery and its key adaptor during cytokinesis have been studied intensely. Cep55 localizes to centrosomes prior to mitosis and is then distributed onto the mitotic spindle and the spindle midzone microtubules, before it accumulates at the midbody as cells divide (Zhao et al., 2006). During interphase, ESCRT-I accumulates with Cep55 at centrosomes (Morita et al., 2007b, 2010; Xie et al., 1998). In contrast, detectable levels of ESCRT-I appear at the midbody well after Cep55 has been targeted to the site (Elia et al., 2011). Super resolution-based imaging of ESCRT-I places it at the cortical surface of the midbody, organized in two hollow rings that adhere to the inner leaflet of the plasma membrane (Elia et al., 2011). Alix, which also binds to Cep55, is distributed in a similar manner (Elia et al., 2011; Morita et al., 2007b). These data are consistent with a role for Cep55 in recruitment of the ESCRT machinery, although their limited overlap suggests additional regulation of ESCRT-I distribution, potentially by components of the midbody ring.

Assembly of Vps32 occurs subsequently to ESCRT-I arrival, in similar membrane-associated rings, which only partially overlap with ESCRT-I containing structures (Elia et al., 2011). Between 15 and 20 minutes prior to membrane scission, a second population of Vps32 appears ~1 μm away from the midbody, which marks the site of abscission and exhibits additional constriction relative to the remaining intracellular bridge. An equivalent accumulation of Vps32 occurs on the opposite side of the midbody, although its appearance is not simultaneous with the first event (Lafaurie-Janvore et al., 2013; Elia et al., 2011). In a similar manner, several other ESCRT-III subunits function near the midbody during this time period, including Vps24, Vps2, Ist1 and Did2 (Dukes et al., 2008; Morita et al., 2010). In particular, the Did2 MIM domain has been shown to bind to the MIT domain of spastin, which cleaves microtubules within the intracellular bridge to facilitate membrane scission (Roll-Mecak & Vale, 2008; Yang et al., 2008). The appearance of Vps4, ~10 minutes prior to each scission event, coincides with the release of the midbody on one side, followed by the other (Elia et al., 2011; Morita et al., 2010). In many cases, the midbody ring is internalized by one of the daughter cells and ultimately degraded within lysosomes (Kuo et al., 2011; Pohl & Jentsch, 2008). Thus, in many respects, the midbody represents an atypical cargo of the ESCRT pathway.

Mechanisms by which the multiple distinct Vps32 populations arise during abscission are unclear. As physical links between these accumulations have been difficult to resolve, it is not yet known whether ESCRT-I and/or Alix could directly initiate ESCRT-III polymerization at constriction sites. Based on tomographic reconstruction of the intracellular bridge in mammalian tissue culture cells, ~17 nm thick spiral filaments have been observed near sites of constriction that are adjacent to the midbody (Guizetti et al., 2011). However, the composition of these structures is unknown. As discussed earlier, Vps32 filaments generated in vivo and in vitro range in diameter from ~5 nm to ~9 nm, respectively (Hanson et al., 2008; Henne et al., 2012). Thus, the spirals visualized by tomography are unlikely to correspond to Vps32 filaments, unless they laterally associate or interact with other protein polymers. Alternatively, the spirals could correspond to distinct ESCRT-III polymers, which have been shown to form helical tubes and may be nucleated by Vps32 (Bajorek et al., 2009; Bodon et al., 2011; Lata et al., 2008b) (Figure 7). It is also important to emphasize that several other factors implicated in cytokinesis can form filaments that bind to membranes, including the septin family of proteins (Field et al., 1996; Lukoyanova et al., 2008). Notably, depletion of Septin 9 results in a defect in abscission that is similar to that
observed following inhibition of ESCRT-III (Estey et al., 2010). Direct interactions between ESCRT components and septins have not been described. However, the associations may be tightly regulated or require several contact points between multiple subunits to co-assemble. The precise composition of the spiral filaments that form at constriction sites will need to be deciphered to understand their contribution to abscission.

In contrast to other ESCRT-dependent scission events, in which the arrival of ESCRT-III and the ATPase complex coincides with the rapid completion of membrane remodeling, cytokinetic abscission occurs ~10–20 minutes after the accumulation of ESCRT-III and ESCRT-IV within the intracellular bridge (Elia et al., 2011). These data suggest that regulatory checkpoints that ensure the faithful completion of cytokinesis may exist. By measuring pulling forces between cells during division, tension was found to regulate the timing of abscission (Lafaurie-Janvore et al., 2013). Only when tension was relieved did the daughter cells become fully isolated. The mechanism by which tension is sensed remains unclear, but may involve the Aurora B kinase. During mitosis, Aurora B-mediated phosphorylation destabilizes connections between microtubules that direct chromosome segregation, and kinetochores, which associate directly with DNA (Liu et al., 2009). In the absence of this tension sensing mechanism, chromosome biorientation fails, resulting in chromosome missegregation and aneuploidy. During abscission, Aurora B has been shown to phosphorylate a paralog of Vps32 called CHMP4C, which redirects its localization to the midbody, where both ESCRT-I and Alix localize (Carlton et al., 2012). Here, phosphorylated CHMP4C inhibits abscission, potentially by impairing the polymerization of other ESCRT-III subunits. It remains to be shown whether Aurora B responds to membrane tension within the intracellular bridge to regulate CHMP4C activity. However, these findings collectively highlight yet another distinction between ESCRT-mediated membrane scission during cytokinesis and ILV budding.

Concluding perspectives

Although the ESCRT machinery is conserved from archaeal species to humans, numerous adaptations have occurred to enable its function in both endolysosomal protein trafficking and cell division (Table 2). The minimal set of ESCRT proteins that can drive membrane scission includes components of ESCRT-III and ESCRT-IV. The locations to which these components are recruited appear to have evolved separately. In archaea, the unique filament forming protein CdvA plays a key role in recruiting ESCRT-III to enable cell division (Dobro et al., 2013). Although CdvA does not exhibit the ability to constrict, the need for this property is rendered moot, as archaeal cells are typically no more than 1 μm in diameter, which appears to be within the capability of ESCRT-III filaments to taper. Budding yeast failed to evolve a recruitment factor for ESCRT-III at the cell division plane, rendering it dispensable for cytokinesis. However, new adaptors enabled ESCRT-III and ESCRT-IV to function in the sorting of integral membrane proteins. To do so, additional machinery was required to capture these molecules (largely ESCRT-0) and bend the endosomal membrane (potentially ESCRT-II:Vps20) to sequester cargoes away from the cytoplasm, thereby terminating their ability to interact with downstream effectors. In this manner, the ESCRT pathway serves as a key tumor suppression system, restricting constitutive signaling that is often a hallmark of cancer (Li & Cohen, 1996; Thompson et al., 2005; Vaccari & Bilder, 2005). Notably, in cases where the ESCRT machinery is required for viral budding, ESCRT-0, ESCRT-II and Vps20 are all dispensable, as cargo capture and membrane bending processes are driven by virally-encoded factors (Langelier et al., 2006; Morita et al., 2011; Sandrin & Sundquist, 2013; von Schwedler et al., 2003). Although plants evolved similar machinery to sort ubiquitin-modified cargoes in the endolysosomal system, an alternative set of cargo adaptors were developed (e.g. the Tom1 family) as compared to yeast and animal cells. These key evolutionary steps help to define how each component of the ESCRT machinery participates in protein trafficking and cytokinetic abscission.

Given their distinctive ability to facilitate membrane scission in a topologically unique manner as compared to the actions of large and small GTPases implicated in other vesicle budding events, it is perhaps not surprising that the ESCRT machinery is hijacked under several forms of viral infection. Successful budding of enveloped viruses requires a scission event at the plasma membrane, which must either be orchestrated by virally encoded proteins (e.g. influenza encoded M2) or host factors (Morita et al., 2011; Rossman et al., 2010). These observations strongly suggest that additional roles for the ESCRT machinery in other virally-induced membrane remodeling events are likely to be
Table 2. Cellular activities of different ESCRT complexes in a variety of species.

<table>
<thead>
<tr>
<th>Cellular activity</th>
<th>ESCRT complex implicated</th>
<th>Species observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor trafficking</td>
<td>ESCRT-0</td>
<td>S. cerevisiae, C. elegans, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td>(e.g. EGFR, Notch receptor, LDL receptor, wingless, frizzled, etc.)</td>
<td>ESCRT-I</td>
<td>A. thaliana, S. cerevisiae, C. elegans, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-II</td>
<td>S. cerevisiae, C. elegans, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-III</td>
<td>A. thaliana, S. cerevisiae, C. elegans, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-IV</td>
<td>A. thaliana, S. cerevisiae, C. elegans, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td>Cell division (e.g. abscission)</td>
<td>ESCRT-0</td>
<td>Not observed</td>
</tr>
<tr>
<td></td>
<td>ESCRT-I</td>
<td>A. thaliana, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-II</td>
<td>D. melanogaster, H. sapiens</td>
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<td></td>
<td>ESCRT-III</td>
<td>D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-IV</td>
<td>D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td>Macroautophagy</td>
<td>ESCRT-0</td>
<td>C. elegans, H. sapiens</td>
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<tr>
<td></td>
<td>ESCRT-I</td>
<td>D. melanogaster, H. sapiens</td>
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<tr>
<td></td>
<td>ESCRT-II</td>
<td>D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-III</td>
<td>A. thaliana, C. elegans, D. melanogaster, H. sapiens</td>
</tr>
<tr>
<td></td>
<td>ESCRT-IV</td>
<td>D. melanogaster, H. sapiens</td>
</tr>
</tbody>
</table>

Although the function of the ESCRT machinery is largely conserved, the roles of the ESCRT complexes in different species vary, which is summarized here. Studies using S. cerevisiae (budding yeast), C. elegans (nematodes), D. melanogaster (insects), H. sapiens (human cells), S. acidocaldarius (archaea), and A. thaliana (plants) are described. Please refer to the text for references.

In summary, this review has highlighted the substantial progress made in the ESCRT field over the past several years. However, we have also laid out the major outstanding questions that remain regarding the mechanisms by which the ESCRT machinery acts. By addressing these issues, our understanding of endosomal sorting and cytokinesis will be dramatically improved, and enable a better comprehension of disease states that arise as a result of ESCRT dysfunction.

**Declarations of interest**

This work was supported by a grant from the NIH: GM088151 to AA.

**References**

- Babst M, Wendland B, Estepe EJ, Emr SD. (1998). The Vps4p AAA ATPase regulates membrane association of a Vps protein inhibit the macroautophagy pathway (Jones et al., 2012). However, the development of new assays or reconstitution-based approaches should resolve this issue in the near future. This area of research is further fueled by the recent implication of the ESCRT machinery in several neurodegenerative diseases, in which mutations in ESCRT components appear to cause defects in autophagy (Lee & Gao, 2009; Lee et al., 2007).


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