No strings attached: the ESCRT machinery in viral budding and cytokinesis

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Summary

Since the initial discovery of the endosomal sorting complex required for transport (ESCRT) pathway, research in this field has exploded. ESCRT proteins are part of the endosomal trafficking system and play a crucial role in the biogenesis of multivesicular bodies by functioning in the formation of vesicles that bud away from the cytoplasm. Subsequently, a surprising role for ESCRT proteins was defined in the budding step of some enveloped retroviruses, including HIV-1. ESCRT proteins are also employed in this outward budding process, which results in the resolution of a membranous tether between the host cell and the budding virus particle. Remarkably, it has recently been described that ESCRT proteins also have a role in the topologically equivalent process of cell division. In the same way that viral particles recruit the ESCRT proteins to the site of viral budding, ESCRT proteins are also recruited to the midbody – the site of release of daughter cell from mother cell during cytokinesis. In this Commentary, we describe recent advances in the understanding of ESCRT proteins and how they act to mediate these diverse processes.

This article is part of a Minifocus on the ESCRT machinery. For further reading, please see related articles: 'The ESCRT machinery at a glance' by Thomas Wollert et al. (*J. Cell Sci*. **122**, 2163-2166) and 'How do ESCRT proteins control autophagy?' by Tor Erik Rusten and Harald Stenmark (*J. Cell Sci*. **122**, 2179-2183).

Key words: Abscission, Cytokinesis, ESCRT, HIV-1, L-domain, Retroviral assembly

Introduction

The endosomal sorting complex required for transport (ESCRT) proteins were initially identified in *Saccharomyces cerevisiae* as class E vacuolar protein sorting (*Vps*) gene products (Katzmann et al., 2002; Raymond et al., 1992). Since then, the majority of work aimed at characterising these proteins has focused on determining their function in the sorting and degradation of ubiquitylated membrane receptors in the late endosome-lysosome pathway. Several models have been proposed to explain the association of the different complexes and the mechanics of membrane deformation and vesicle formation (Hurley and Emr, 2006; Nickerson et al., 2007). Surprisingly, work carried out more recently has revealed additional functions of the ESCRT proteins in viral budding and cytokinesis. Now, the question to address is: what aspects of ESCRT function are required for these processes? It is not known whether viral budding requires the same membrane deformation activity as is involved in the biogenesis of multivesicular bodies (MVBs), or whether this function is provided by the oligomerisation of the membrane-associated viral Gag proteins (Gottlinger, 2001). Likewise, during cytokinesis, the daughter cell is formed by massive membrane remodelling, involving the contraction of an actomyosin ring around the cell perimeter, rather than the 'budding' of a nascent cell from the plasma membrane. Despite differences in the processes of MVB biogenesis, viral-particle release and cytokinesis, it is clear that they have in common the requirement for a membrane-scission event for the release of the progeny vesicle, virion or daughter cell (Fig. 1). Here, we explore the evidence supporting the role of ESCRT proteins in membrane scission and the possibility that different ESCRT subunits are required for differential ESCRT functions.

From yeast to humans – assembly and function of the ESCRT machinery

The ESCRT proteins make up four protein complexes termed ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III. This machinery assembles on the endosomal membrane and, through a series of ubiquitin-interacting elements, identifies ubiquitin-labelled receptors, which are destined for lysosomal degradation. Sorting of these receptors into the intralumenal vesicles (ILVs) of MVBs allows subsequent exposure to lysosomal acidic hydrolases when the MVBs fuse with the lysosome. Studies in yeast suggest that the ESCRTs are sequentially recruited to the endosomal membrane through various protein and lipid interactions (Burd and Emr, 1998; Gill et al., 2007b; Katzmann et al., 2003; Stahelin et al., 2002). Recent structural studies have provided a better understanding of how ESCRT-I and ESCRT-II are assembled, and of their interactions with ubiquitylated cargo (Gill et al., 2007a; Kostelansky et al., 2007; Kostelansky et al., 2006; Pineda-Molina et al., 2006; Teo et al., 2006; Teo et al., 2004a; Teo et al., 2004b), areas that have been expertly reviewed (Hurley and Emr, 2006; Williams and Urbe, 2007). Finally, ESCRT-III recruits the AAA-ATPase Vps4, a key enzyme that is required for ESCRT function and disassembly. Although ESCRT proteins and the protein-protein interactions of the ESCRT pathway are remarkably conserved from yeast to humans (Bowers et al., 2004; Martin-Serrano et al., 2003b; von Schwedler et al., 2003), some differences are evident. For example, ESCRT-II is required for MVB biogenesis in yeast, but its functional significance in humans is still under debate (Bowers et al., 2006; Langelier et al., 2006; Malerod et al., 2007).

The core ESCRT machinery

ESCRT-III is thought to provide the core activities of membrane deformation and scission, and is required for all known ESCRT

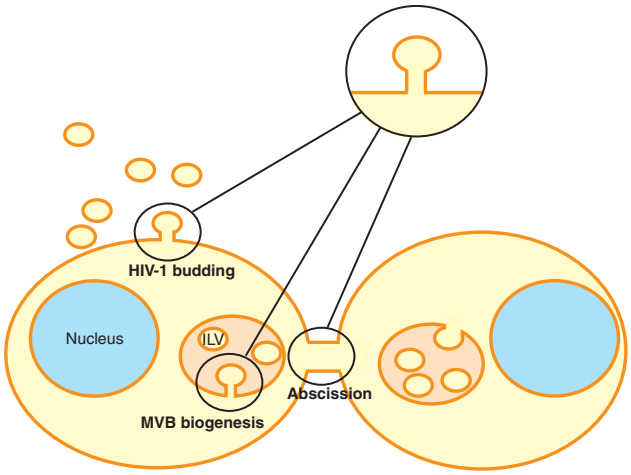

Fig. 1. A diagram illustrating topologically equivalent 'budding' events for which the ESCRT machinery is required. The formation of intralumenal vesicles (ILVs) within multivesicular bodies (MVBs), viral budding and abscission during cytokinesis all require the resolution of a cytoplasm-filled membranous tether.

functions in yeast and humans. Some of the pioneering work in this field suggested that ESCRT-III assembles as an insoluble lattice on the endosomal membrane and mediates membrane deformation through a mechanism involving Vps4. Deletion of ESCRT-III components, or the expression of catalytically inactive Vps4 mutants, prevents the normal degradation of endosomal cargo and causes the accumulation of aberrant endosomes (Babst et al., 2002; Babst et al., 1997; Babst et al., 1998; Bishop and Woodman, 2000). A function in membrane scission is also suggested by the finding that these large aberrant endosomes lack internal vesicles (Babst et al., 1997; Odorizzi et al., 1998).

Human ESCRT-III consists of charged MVB protein (CHMP) subunits, some of which have evolved multiple isoforms (see Table 1). Studies in yeast indicate that these proteins are arranged into two subcomplexes, one that contains CHMP4A, CHMP4B or CHMP4C (yeast Snf7; yeast nomenclature will hereafter be shown in parentheses unless otherwise indicated) and CHMP6 (Vps20), and another that consists of CHMP2A or CHMP2B (Vps2) and CHMP3 (Vps24) (Babst et al., 2002). CHMP1A, CHMP1B (Vps46, Did2) and CHMP5 (Vps60) play important accessory and regulatory roles in ESCRT function (Azmi et al., 2008; Lottridge et al., 2006; Nickerson et al., 2006). The recently identified human protein CHMP7 (of which there is no known yeast homolog) interacts with CHMP4 proteins (Horii et al., 2006), although further investigations are required to determine its function.

The CHMP proteins are similar in form to one another, each with an N-terminal basic region and a C-terminal acidic region. Structural studies of CHMP3 suggest that CHMP proteins have an autoinhibitory activity whereby the uncomplexed protein folds into a closed conformation through the attractive charges. However, following activation, the protein conformation may 'open', enabling CHMP proteins to associate with each other, forming a lattice on the membrane (Lata et al., 2008a; Muziol et al., 2006; Shim et al., 2007; Zamborlini et al., 2006). It has been proposed that the N-terminal basic region associates with the membrane via electrostatic charges with the lipid bilayer (Muziol et al., 2006), although additional anchorage may be provided by phosphoinositide-binding domains found in CHMP3 (Whitley et al., 2003) and CHMP4 (Lin et al., 2005) and a myristyl group in CHMP6 (Yorikawa et al., 2005). However, the C-terminal domains are free to recruit additional ESCRT-binding partners. CHMP proteins bind to microtubule-interaction and trafficking (MIT)-domain-containing proteins, such as VPS4 and the activator protein LIP5, via a MIT-interaction motif (MIM) at their C-termini (Agromayor and Martin-Serrano, 2006; Azmi et al., 2008; Nickerson et al., 2006; Obita et al., 2007; Scott et al., 2005b; Shim et al., 2008; Stuchell-Brereton et al., 2007; Tsang et al., 2006; Xiao et al., 2008). The connection between the VPS4-LIP5 complex and the CHMP4-CHMP6 subcomplex occurs via a slightly different mechanism, as these CHMP proteins do not contain the conventional MIM. Instead, Kieffer et al. have reported an alternative interaction that occurs between CHMP6 and the MIT domain of VPS4 through a region of CHMP6 that they term an 'MIM2 element' (Kieffer et al., 2008). Functional MIM2 elements have also been identified in CHMP4 and the regulatory protein IST homolog (hIST1) (described below) (Bajorek et al., 2009).

VPS4 enzymes form oligomeric complexes on endosomal membranes (Babst et al., 1998; Scott et al., 2005a); these complexes are comprised of two hexameric (Yu et al., 2008) or heptameric (Hartmann et al., 2008) rings. In yeast, Vta1 (human LIP5) stimulates the ATPase activity of Vps4 (Azmi et al., 2006; Lottridge et al., 2006) and promotes the assembly of the double ring structure (Xiao et al., 2008). VPS4 activity is also regulated by several proteins, including ESCRT-III itself (Azmi et al., 2008) and the newly identified ESCRT-related protein Ist1 (Dimaano et al., 2008; Rue et al., 2008). Ist1 forms a subcomplex with Did2 (human CHMP1A or CHMP1B) and, in humans, hIST1 interacts with LIP5, VPS4 and other MIT-domain-containing proteins via two MIMs located at the extreme C-terminus (Agromayor et al., 2009; Bajorek et al., 2009). Studies in yeast initially suggested that the Ist1-Did2 complex is a positive modulator of Vps4 through the stabilisation of its interaction with ESCRT-III at the endosomal membrane (Rue et al., 2008). Additionally, negative regulation of Vps4 by Ist1 in the cytoplasm has also been proposed (Dimaano et al., 2008). In summary, ESCRT-III proteins form an insoluble lattice on the endosomal membrane via N-terminal interactions, while their C-terminal domains act to recruit the AAA-ATPase VPS4 and other regulatory proteins that are required for function.

A membrane-deformation and -scission machinery

The formation of ILVs within MVBs during cargo sorting requires the coordinated deformation of the endosomal membrane to form a nascent vesicle, followed by a membrane-scission event that releases the ILV into the MVB. The idea that ESCRT-III proteins were involved in membrane deformation was initially suggested by the formation of filamentous polymers on the plasma membrane by overexpressed CHMP4A and CHMP4B: surprisingly, the filaments formed circular arrays that were capable of deforming the membrane (Hanson et al., 2008). In agreement with these observations, Snf7 (human CHMP4) and Vps24 (human CHMP3) form helical polymers in vitro that can be disassembled by Vps4 in the presence of ATP. A role for Vps4 in the assembly of the ESCRT-III lattice is also suggested by the finding that, in the presence of ADP, Vps4 causes the chimeric filaments to bundle into extensive cables (Ghazi-Tabatabai et al., 2008). Recent work by Scott Emr and colleagues has also provided genetic and biochemical evidence that supports the idea that individual ESCRT-III subunits have specific functions and are recruited in an ordered manner. Specifically, binding of Vps20 (human

CHMP6) to the membrane initiates the oligomerisation of Snf7 subunits to form filaments at the MVBs. Subsequently, these filaments could encircle and concentrate endosomal cargo into defined regions of membrane. The oligomerisation reaction would then be terminated by the binding of the Vps24-Vps2 (human CHMP3-CHMP2) subcomplex, which also recruits Vps4 (Saksena et al., 2009; Teis et al., 2008).

A model has also been proposed for ESCRT disassembly: it has been suggested that individual subunits are 'pumped' through the central pore of the VPS4 complex into the cytoplasm (Scott et al., 2005a; Stuchell-Brereton et al., 2007; Yu et al., 2008). In line with the mechanism of function for spastin, another MIT-domain-containing AAA-ATPase (Roll-Mecak and Vale, 2008; White et al., 2007), the ESCRT-III subunits could be moved to binding sites within the central pore of the AAA-ATPase ring (Gonciarz et al., 2008; Shim et al., 2008), before being 'pulled' through via conformational changes induced during ATP binding and hydrolysis. It has been suggested that removing the subunits in this fashion could result in a constricting force that promotes vesicle extrusion and neck closure (Kieffer et al., 2008).

A direct role for ESCRT-III in membrane scission has recently been demonstrated by reconstituting this activity using an in vitro system of MVB formation (Wollert et al., 2009a), and the mechanistic details of this reaction have also started to be elucidated. Recent in vitro experiments conducted with C-terminally truncated CHMP2A (CHMP2AΔCTD) and CHMP3 have demonstrated that these proteins can oligomerise and assemble into long tubular structures. Intriguingly, the coexpression of CHMP2AΔCTD, CHMP3 and VPS4 results in the formation of tubular structures in which VPS4 is on the inside of the tube, whereas the membrane-interaction sites of the CHMP proteins are on the outside. Importantly, the tubular arrays of ESCRT proteins had a diameter in the range of that seen for MVB-vesicle and virus-particle stalks, suggesting that these tubular structures might be important for membrane scission. Interestingly, the addition of ATP caused the disassembly of the tubes (Lata et al., 2008b). It is possible that extraction of ESCRT-III subunits through a VPS4 ring could pull the membrane together, leading to the resolution of the membrane stalk. Thus, in a similar way that the enzyme dynamin forms a ring around inwardly budding vesicles, perhaps VPS4 – the only known energy-expending protein of the ESCRT pathway – acts on the internal membrane of an outwardly budding vesicle (Fig. 2). It should be noted that the tubular structures described by Lata et al. differ significantly from the helical filaments reported by Ghazi-Tabatabai et al., although the reason for this difference is unclear (Lata et al., 2008b; Ghazi-Tabatabai et al., 2008). One intriguing possibility that might explain these conflicting results is that the two conformations might represent sequential stages of the ESCRT-III oligomeric structures that are required for sorting and membrane-scission events. However, it is important to take into account that these structures do not comprise all four ESCRT-III proteins, and recent evidence has shown that at least Snf7, Vps24 and Vps20 are required for MVB formation (Wollert et al., 2009a).

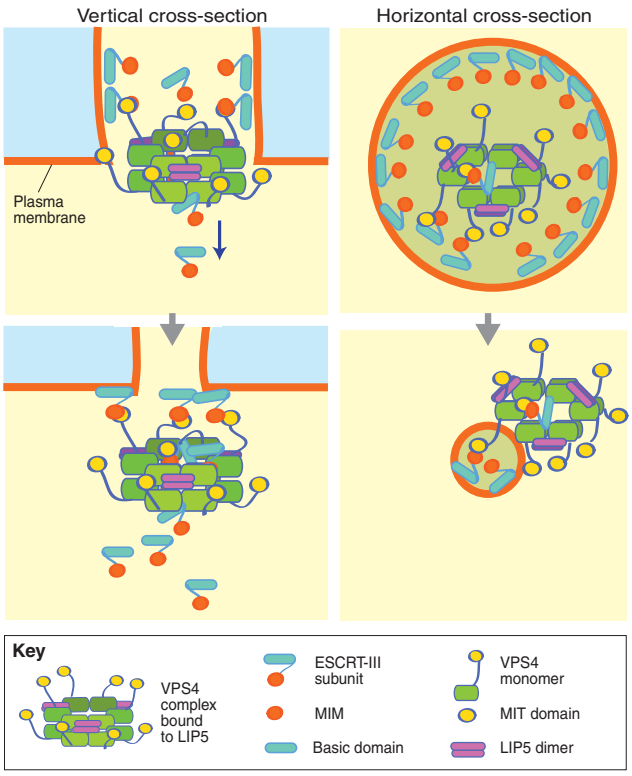

Fig. 2. A model for ESCRT-III–VPS4-mediated membrane scission. Following recruitment by upstream ESCRT complexes, ESCRT-III assembles on the membrane. N-terminal basic domains (blue) of ESCRT-III subunits attach to the membrane, whereas the flexibly linked C-terminal MIMs (MIT-interaction motifs; red) project away from the membrane. The AAA-ATPase VPS4 assembles into a double-ring complex and interacts with ESCRT-III subunits via N-terminal MIT (microtubule interaction and trafficking) domains (yellow). The regulatory protein LIP5 (purple) forms a dimer and associates with the VPS4 complex (note that the MIT domains of LIP5 have been omitted for clarity). Narrowing of the membrane tube might be concurrent with VPS4-mediated disassembly of the ESCRT-III lattice. It is possible that subunit removal via mechanical extraction through the VPS4 complex could reduce the diameter of the tube and lead to constriction. Top two panels show vertical and horizontal cross-sections of a wide membrane tube. The bottom two panels illustrate cross-sections of the thinner tube formed following the removal of the ESCRT-III lattice from the wider tube.

ESCRTs and virus-particle release

ESCRTs and virus budding

A short time after the ESCRT 'budding' machinery was initially discovered, a surprising new role for these proteins was defined in the release of enveloped retroviruses from the host cell. This discovery was preceded by the finding that the release of HIV-1 particles could be blocked by the mutation of four amino acids (P_7TAP_{10}) in the p6 protein of the HIV-1 Gag polyprotein (Gottlinger et al., 1991; Huang et al., 1995). Mutation of the PTAP motif or truncation of the p6 protein resulted in fully formed viral particles that remained attached to the plasma membrane of the host cell by a membranous tether. Several other enveloped viruses from the filovirus and rhabdovirus families also appear to contain short peptide motifs that are essential for virus release at a late stage of assembly and budding (Craven et al., 1999; Harty et al., 2000; Harty et al., 1999). The term late domain (L-domain) was thus coined to describe these motifs.

The idea that viral L-domains were recruiting a host cell factor(s) was supported by several findings. First, L-domains can function in a positionally independent manner within viral Gag proteins (Parent et al., 1995). Second, L-domains are interchangeable between different viral Gag proteins (Parent et al., 1995; Yuan et al.,

Table 1. Interactions between and requirement for ESCRT subunits in MVB biogenesis, viral-particle release and cytokinesis in mammalian cells

Subunit*	Known interaction partners	Role in: MVB biogenesis	Viral budding	Cytokinesis
ESCRT-0				
HRS	Tsg101, STAM, PtdIns(3)*P*, ubiquitin, SNAP25, VPS37A, clathrin, Cep55	Required for EGFR and EGF degradation[1,2]	Not required[3]	Recruited to midbody[4]
STAM1 and STAM2	HRS, UBPY, ubiquitin, AMSH	Required for EGFR degradation[5]	N.D.	N.D.
ESCRT-I				
Tsg101 (Vps23)	All ESCRT-I subunits, EAP30, EAP45, ALIX, HRS, Bcr, TOM1, TOM1L1, TOM1L2, ubiquitin, Cep55, Rock1, IQGAP, CD2AP, HD-PTP	Recruited by HRS to endosomal membrane; required for EGFR and EGF degradation[2,6-9]	Recruited to plasma membrane by HIV-1 Gag to mediate virus budding[10,11]	Recruited to midbody by Cep55 to mediate abscission[12,13]
VPS28-I and VPS28-II	Tsg101, CHMP6, Bcr, ESCRT-II	Blocking antibody inhibits EGF degradation[8]	Required[14-16]	Required[12]
VPS37A, B, C, D	Tsg101, Cep55; VPS37A binds to HRS	VPS37A required for EGFR degradation[17]	VPS37B and VPS37C required for HIV-1 release[16,18]	N.D.
MVB12A, MVB12B	Tsg101; MVB12A binds to VPS37B	Recruited to aberrant endosomes[19]	Role in maturation and/or infectivity of HIV-1[19]	N.D.
ESCRT-II				
EAP30, EAP20, EAP45	CHMP6, VPS28-I, ubiquitin, Tsg101, with one another	May be required for EGFR degradation; not required for MHC-1 degradation[20,21]	Not required[20]	N.D.
ESCRT-III				
CHMP1A and CHMP1B (Did2) – contain MIM	MITD1, UBPY, LIP5, AMSH, hIST1, VPS4, with one another; CHIMP1B binds to CHMP2A, CHMP4B, CHMP5, spastin	GFP fusion causes aberrant endosomes[22]	Overexpressed YFP fusion inhibits budding[23]	Required for abscission[24]
CHMP2A and CHMP2B (Vps2) – contain MIM	CHMP2A binds to AMSH, MITD1, VPS4, CHMP1B, CHMP3, CHMP4A, CHMP4B, CHMP5; CHMP2B binds to CHMP3	Expression of truncated form causes aberrant endosomes[25]	CHMP2A-YFP fusion inhibits budding; CHMP2B has no DN effect[26,27]	CHMP2A recruited to midbody[13]
CHMP3 (Vps24)	CHMP2A, CHMP2B, CHMP4B, VPS4, AMSH	Required for proper EGFR degradation[28]	Overexpressed YFP fusion inhibits budding[26]	Abscission inhibited by DN mutant[29]
CHMP4A, B, C (Snf7) – contain MIM2	CHMP6, ALIX, Brox, HD-PTP, VPS4, with one another; CHMP4B binds to CHMP1B, CHMP3, CHMP5, CHMP7; CHMP4C binds to AMSH, UBPY	Expression of a truncated form causes aberrant endosomes[25]	YFP fusion proteins inhibit budding[26,27,30]	Required for abscission[30,13]
CHMP5 (Vps60)	CHMP1B, CHMP2A, CHMP4A, CHMP4B, LIP5	Required for proper EGFR and TGFβR degradation[31,32]	YFP fusion was shown to inhibit, but depletion enhanced, virus release[27,31]	Recruited to midbody[13]
CHMP6 (Vps20) – contains MIM2	CHMP4A, CHMP4B, EAP45, EAP20, EAP30, VPS28-I, VPS4	Required for proper EGFR degradation[20]	Not required[20]	N.D.
CHMP7	CHMP4B	GFP fusion inhibits EGFR degradation[33]	GFP fusion inhibits VLP release[33]	N.D.
ESCRT-associated proteins				
ALIX	Tsg101, endophilin, CHMP4A, CHMP4B, CHMP4C, Cep55, CD2AP, Cin85	Not essential for EGFR degradation[34,35]	Required for EIAV budding; minor activity in HIV-1 release[36,27]	Recruited by Cep55 to midbody to mediate abscission[12,13]

Table 1 continued on next page.

Table 1. Continued

Subunit*	Known interaction partners	Role in: MVB biogenesis	Role in: Viral budding	Role in: Cytokinesis
ESCRT-associated proteins				
LIP5 – contains MIT domains	CHMP1A, CHMP1B, CHMP5, VPS4, hIST1	Required for proper EGFR degradation[31]	Required for HIV-1 release[31]	Binding partners are required[24,40]
VPS4A, VPS4B – contain MIT domain	CHMP1A, CHMP1B, CHMP2A, CHMP2B, CHMP3, CHMP4A, CHMP4B, CHMP4C, LIP5, hIST1	Required for dissociation of complexes from endosomal membrane[37]	Required for viral budding[11,26]	Abscission inhibited by DN mutant[13]
AMSH – contains MIT domain	CHMP1A, CHMP1B, CHMP2A, CHMP3, CHMP4C, STAM, hIST1	Depletion increases rate of EGFR degradation[38]	Not essential[23]	Recruited to midbody; depletion causes moderate defect[4]
UBPY – contains MIT domain	CHMP1A, CHMP1B, CHMP4C, STAM, hIST1	Required for deubiquitylation of EGFR[39]	N.D.	Catalytic mutant inhibits abscission; recruited to midbody[4]
hIST1 – contains MIM1 and MIM2	CHMP1A, CHMP1B, MITD1, spastin, VPS4, LIP5, AMSH, UBPY	Not required for EGFR degradation[40]	Not essential[24,40]	Required for abscission[24,26]

*Yeast nomenclature is shown in parentheses.

Abbreviations not included in main text: DN, dominant-negative; EGFR, epidermal growth factor receptor; EIAV, equine infectious anaemia virus; GFP, green fluorescent protein; N.D. not determined; PtdIns(3)*P*, phosphatidylinositol 3-phosphate; TGFβR, transforming growth factor-β receptor; VLP, virus-like particle; YFP, yellow fluorescent protein.

[1](Urbe et al., 2003); [2](Raiborg et al., 2008); [3](Pornillos et al., 2003); [4](Mukai et al., 2008); [5](Kanazawa et al., 2003); [6](Bache et al., 2003); [7](Katzmann et al., 2003); [8](Bishop et al., 2002); [9](Babst et al., 2000); [10](Martin-Serrano et al., 2001); [11](Garrus et al., 2001); [12](Carlton and Martin-Serrano, 2007); [13](Morita et al., 2007b); [14](Martin-Serrano et al., 2003a); [15](Tanzi et al., 2003); [16](Stuchell et al., 2004); [17](Bache et al., 2004); [18](Eastman et al., 2005); [19](Morita et al., 2007a); [20](Langelier et al., 2006); [21](Bowers et al., 2006); [22](Howard et al., 2001); [23](Agromayor and Martin-Serrano, 2006); [24](Bajorek et al., 2009); [25](Shim et al., 2007); [26](von Schwedler et al., 2003); [27](Martin-Serrano et al., 2003b); [28](Bache et al., 2006); [29](Dukes et al., 2008); [30](Carlton et al., 2008); [31](Ward et al., 2005); [32](Shim et al., 2006); [33](Horii et al., 2006); [34](Schmidt et al., 2004); [35](Cabezas et al., 2005); [36](Strack et al., 2003); [37](Babst et al., 1998); [38](McCullough et al., 2004); [39](Row et al., 2007); [40](Agromayor et al., 2009).

2000) and hence are not virus specific. Additionally, a number of studies have also implicated an aspect of the ubiquitylation machinery in viral budding: proteasome inhibitors were shown to cause an L-domain-defective phenotype in cells infected with HIV-1 or Rous sarcoma virus, or that were expressing Simian immunodeficiency virus type (SIV_{mac}) Gag (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000). In 2001, the search for the HIV-1 'budding factor' ended when the interaction between the PTAP motif of HIV-1 p6 and the ESCRT-I protein Tsg101 was shown, with the demonstration that Tsg101 recruits the entire ESCRT machinery to mediate virus budding (Demirov et al., 2002; Garrus et al., 2001; Martin-Serrano et al., 2001; VerPlank et al., 2001). This finding sparked a flood of interest in the field and, since then, several other viruses, including those from the arenavirus and paramyxovirus families, have also been found to contain L-domains (Bieniasz, 2006; Perez et al., 2003; Schmitt et al., 2005). Interestingly, the requirement of a functional ESCRT pathway for particle release is not restricted to RNA viruses. Recent work has demonstrated that the enveloped DNA viruses hepatitis B and herpes simplex virus type 1 also require the ESCRT machinery, because the expression of dominant-negative forms of CHMP3, CHMP4B and CHMP4C or of catalytically inactive VPS4 proteins inhibits virus release (Crump et al., 2007; Lambert et al., 2007; Watanabe et al., 2007).

Although PTAP (and PSAP) L-domains recruit the ESCRT machinery via Tsg101, other L-domains access the complexes through different means. $LYPx_nL$ L-domains bind the adaptor protein ALIX, which in turn binds and recruits the ESCRT machinery via the CHMP4 proteins (Fisher et al., 2007; Martin-Serrano et al., 2003b; McCullough et al., 2008; Strack et al., 2003; Usami et al., 2007; von Schwedler et al., 2003; Zhai et al., 2008). The PPxY motif enlists members of the NEDD4 family of ubiquitin ligases through interactions with WW domains (Bouamr et al., 2003; Garnier et al., 1996; Harty et al., 2000; Harty et al., 1999; Kikonyogo et al., 2001; Martin-Serrano et al., 2005; Yasuda et al., 2002), although the precise link between the NEDD4-like proteins and the ESCRT machinery is still unknown (Martin-Serrano, 2007). Finally, a fourth L-domain encoded by the FPIV amino acid motif has been identified in the matrix protein of the paramyxovirus simian virus 5, although the mechanism of ESCRT recruitment by this L-domain is currently unknown (Schmitt et al., 2005).

Interestingly, many of the viruses studied contain more than one type of L-domain motif (Bouamr et al., 2003; Gottwein et al., 2003; Segura-Morales et al., 2005). For example, HIV-1 contains binding sites for Tsg101 and ALIX (Strack et al., 2003), and, although it lacks a PPxY motif, the surprising influence of NEDD4L (also known as Nedd4-2s) ubiquitin-ligase activity on HIV-1 budding has recently been shown (Chung et al., 2008; Usami et al., 2008). An additional ALIX-binding site has also been identified in the nucleocapsid protein of HIV-1 Gag; this site might play an auxiliary role in virus release (Popov et al., 2008). It is well established that the interaction between the PTAP L-domain motif and Tsg101 is the preferential means of HIV-1 release in HEK293T cells; however, mutation of the PTAP motif renders the virus reliant on its $LYPx_nL$ motif (Fisher et al., 2007; Usami et al., 2007), and, independently, on a region in the C-terminal domain of capsid (CP) and the SP1 region of the Gag polyprotein, for NEDD4L activity (Chung et al., 2008; Usami et al., 2008). The evolution of 'back-up' L-domains illustrates the crucial role of ESCRT proteins and ESCRT-associated cofactors in viral release. However, it is not completely clear why viruses have evolved multiple L-domains. One possibility could be

that differing expression levels of host cofactors require alternative ESCRT entry routes in different cells types. Alternatively, because viral proteins are in competition with other cellular factors for ESCRT adaptor proteins (Pornillos et al., 2003), it is perhaps advantageous for the viral Gag protein to interact with as many ESCRT recruiters as possible.

Other ESCRT functions, in addition to membrane scission

Unlike MVB biogenesis, it is commonly thought that plasma-membrane deformation during retroviral assembly is aided by the concentrated oligomerisation of viral Gag proteins at the membrane. Moreover, the L-domain-defective phenotype suggests that ESCRT proteins are only required to mediate scission in the final release step of viral budding, although an additional early function of the ESCRT machinery in HIV-1 budding has been recently proposed (Carlson et al., 2008). However, in addition to the classic late-budding defects, the mutation of L-domains in some retroviruses, or the depletion or disruption of some ESCRT proteins, can lead to other problems, ranging from early assembly blocks to maturation failure. Observations of cells infected with PPPY L-domain mutants of human T-cell leukaemia virus type 1 and Mason-Pfizer monkey virus by electron microscopy revealed an early-budding phenotype; Gag protein accumulated underneath the plasma membrane, but little or no assembled virus particles were found attached to the cell surface by membranous tethers (Bouamr et al., 2003; Gottwein et al., 2003; Le Blanc et al., 2002). A different phenotype was seen on deletion of the p12 L-domain-containing protein or PPPY motif of murine leukaemia virus; in this viral context, morphologically tubular virions were observed, as well as viral particles that were attached to each other in chains (Yuan et al., 2000). A similar phenomenon also occurred on overexpression of a dominant-negative CHMP2A construct (Martin-Serrano et al., 2003b). Additionally, recent work with the newly identified ESCRT-I proteins MVB12A and MVB12B showed that these proteins have a function in viral infectivity, but not in virus budding. The depletion of MVB12A or MVB12B resulted in the accumulation of amorphous, aberrant viral particles, although particle release was not inhibited. However, the overexpression of these proteins did reduce viral budding, and this was dependent on the ability of the proteins to bind Tsg101 and on their phosphorylation (Morita et al., 2007a). Altogether, these observations suggest that, in addition to a function in membrane scission, some viruses might also require additional aspects of the ESCRT machinery for assembly and/or maturation.

ESCRTs and daughter-cell release

Cytokinesis, the process of cytoplasmic division, is the final stage of the cell cycle following mitosis. It is at this final stage that one cell becomes two. Remarkably, it now seems that the ESCRT machinery is required for a terminal step in this process. In animal cells, the act of cytokinesis is driven by the strictly regulated contraction of an actomyosin ring around the equator of the cell. The position of the contractile ring and the initiation of contraction are determined by signals from the mitotic-spindle microtubules at the spindle midzone (the region of microtubule overlap), and might also be influenced by signals from the aster microtubules at the poles of the cell (Barr and Gruneberg, 2007; Bringmann and Hyman, 2005; Eggert et al., 2006; Glotzer, 2005). The accumulation of actin and myosin-II filaments, together with numerous regulatory proteins, occurs at right angles to the spindle midzone, which ensures the equal distribution of genetic material, organelles and cytoplasm to each progeny cell. When the actomyosin ring contracts, the resulting indentation around the cell is termed the cleavage furrow. Ingression of the furrow ultimately leads to constriction of the plasma membrane around the spindle microtubules and the formation of a condensed midbody structure between mother and daughter cell.

Following the massive plasma-membrane remodelling gymnastics involved in cleavage-furrow ingression, the final hurdle before liberation of the daughter cells is the severing of the membranous tether that joins them. This process, known as abscission, requires the coordinated actions of many proteins that are involved in membrane trafficking, vesicle targeting and vesicle fusion (Fig. 3). Abscission is preceded by the formation of a centriolin-enriched, γ-tubulin-containing 'midbody ring' structure (Gromley et al., 2003; Gromley et al., 2005), to which vesicle-targeting proteins of the exocyst complex are recruited. The exocyst is a large multi-subunit protein complex that functions in the tethering of Golgi-derived secretory vesicles to distinct plasma-membrane regions, and its localisation to the midbody ring seems to be required for cytokinesis (Gromley et al., 2005). Subsequent recruitment to the midbody of endobrevin (a v-SNARE protein; also known as VAMP8) and syntaxin-2 (a t-SNARE protein) by components of the midbody ring facilitates membrane-fusion events that are crucial for abscission (Gromley et al., 2005; Low et al., 2003). Moreover, important regulators of the SNAREs, such as the Rab family of GTPases, have also been implicated in the terminal stages of cytokinesis. Specifically, endocytic Rab proteins (Rab35, Rab11 and, in certain cells, Rab8) are also key regulators of vesicle traffic during the final stages of cell division (Fielding et al., 2005; Kouranti et al., 2006; Pohl and Jentsch, 2008; Wilson et al., 2005; Yu et al., 2007). In addition, the Rab11-family-interacting proteins FIP3 and FIP4 are thought to play key roles in cytokinesis through the transport of Rab11-positive vesicles from recycling endosomes to the cleavage furrow and the midbody (Fielding et al., 2005; Prekeris and Gould, 2008; Wilson et al., 2005).

ESCRT localisation at the midbody

Previous models of cytokinesis proposed that the resolution of the membranous tether between two cells could be achieved by membrane-fusion events that are mediated by SNAREs. According to these models, the combination of membrane addition by vesicle fusion to the plasma membrane, and the influx and fusion of endocytic and secretory vesicles to the midbody region (effectively making 'holes' in the tether) was thought to be sufficient to mediate membrane scission (Gromley et al., 2005). A key activity in this model is the homotypic fusion of vesicles at the midbody in order to facilitate the final event in abscission. However, experimental evidence supporting homotypic fusion during abscission has been elusive. Crucially, the topology of the membrane-scission event that is required for abscission is identical to the topology of the membrane-scission events that are known to be facilitated by the ESCRT machinery – MVB formation and retroviral budding (Fig. 1). This analogy and the functional parallels between these ESCRT-mediated processes have shed light on the cellular mechanisms that facilitate abscission.

In the same way that many enveloped viruses recruit the ESCRT machinery to mediate scission of the membranous tether between virion and host cell, Tsg101 and ALIX are recruited to the midbody to mediate the release of daughter cell from mother cell (Carlton and Martin-Serrano, 2007; Morita et al., 2007b). Cep55 (centrosome protein 55kDa), a midbody component that is crucial for abscission (Fabbro et al., 2005; Martinez-Garay et al., 2006; Zhao et al., 2006),

binds to both Tsg101 and ALIX, and the disruption of this interaction results in cytokinesis failure. It is important to note that the interaction of Tsg101 and ALIX with Cep55 is not required for viral budding (Carlton and Martin-Serrano, 2007; Morita et al., 2007b), suggesting that Cep55 and viral Gag proteins play functionally equivalent roles in recruiting the ESCRT machinery to facilitate topologically equivalent membrane-scission events at different cellular locations.

Importantly, it is probable that other components of the ESCRT machinery are also required for abscission, because the interaction of Tsg101 with VPS28 (a component of ESCRT-I) is also required for the completion of cytokinesis (Carlton and Martin-Serrano, 2007), and interactions between Tsg101 and other proteins that are involved in cytokinesis – in particular CD2AP, ROCK1 and IQGAP – have also been shown (Morita et al., 2007b). The involvement of the core ESCRT machinery in cytokinesis was initially suggested by the dominant-negative effect observed when catalytically inactive VPS4 or ESCRT-III subunits were overexpressed (Carlton and Martin-Serrano, 2007; Dukes et al., 2008; Morita et al., 2007b). Importantly, direct evidence for ESCRT-III function in abscission is provided by the finding that the ESCRT-III-binding site in ALIX has an essential role in this process (Carlton et al., 2008; Morita et al., 2007b).

In terms of localisation, confocal-microscopy studies with immunostaining have uncovered an interesting pattern. On one hand, exogenously expressed CHMP2A, CHMP4A, CHMP5, hIST1 and endogenous VPS4 were found at distinct rings on both sides of the midbody structure (Agromayor et al., 2009; Bajorek et al., 2009; Morita et al., 2007b). By contrast, fluorescently tagged forms of ALIX and Tsg101 were found at the central region of the midbody (Carlton and Martin-Serrano, 2007), suggesting that different components of the ESCRT machinery might localise at different regions of the midbody. However, it is known that protein density at the central region of the midbody masks epitopes, and therefore a gap in this region determined by immunostaining should be interpreted with caution. Evidence that ESCRT proteins constitute a major component of the midbody structure has also been suggested by experiments in which cells were engineered to express ALIX mutants that cannot bind to ESCRT-III. In these cells, aberrant midbodies formed, in which tubulin staining was visible across the central region of interdigitating microtubules. This is in contrast to cells expressing wild-type ALIX, in which a dense accumulation of protein (presumably ESCRT-III) prevented complete tubulin staining and a characteristic gap at the midbody was observed (Carlton et al., 2008). In summary, these observations suggest an organised spatial and temporal recruitment of ESCRT proteins to the midbody during cytokinesis.

Initial mapping studies showed that Cep55 binds to a conserved peptide at the proline-rich region (PRR) of ALIX (Carlton et al., 2008; Morita et al., 2007b). However, the Cep55-binding peptide in ALIX is not found in *Drosophila*, suggesting that different mechanisms to recruit the ESCRT machinery during abscission might exist in other organisms. Biochemical data also indicate that Tsg101 and ALIX compete for binding to the ESCRT- and ALIX-binding region (EABR) of Cep55, and the crystal structure of the ALIX-Cep55 complex shows that the EABR forms a dimeric noncanonical coiled coil that cannot simultaneously bind to Tsg101. On the basis of these observations, a model has been proposed in which multiple Cep55 dimers are required for ESCRT recruitment to the midbody (Lee et al., 2008; Wollert et al., 2009b).

ESCRT function at the midbody

Although the ESCRT proteins appear to be recruited for membrane-scission events, it is formally possible that, rather than having a direct role in cytokinesis, an endosomal sorting function could be the required activity for abscission (Prekeris and Gould, 2008). However, this view is countered by recent discoveries in Archaea, which is a separate domain of life that lacks endomembrane structures and is separated from humans by two-billion years of evolution. Remarkably, homologues of ESCRT-III proteins and VPS4 have been identified in *Sulfolobus*, a member of the Kingdom Crenarchaea (Hobel et al., 2008; Obita et al., 2007). Functional experiments have demonstrated that cell-cycle regulation and mid-

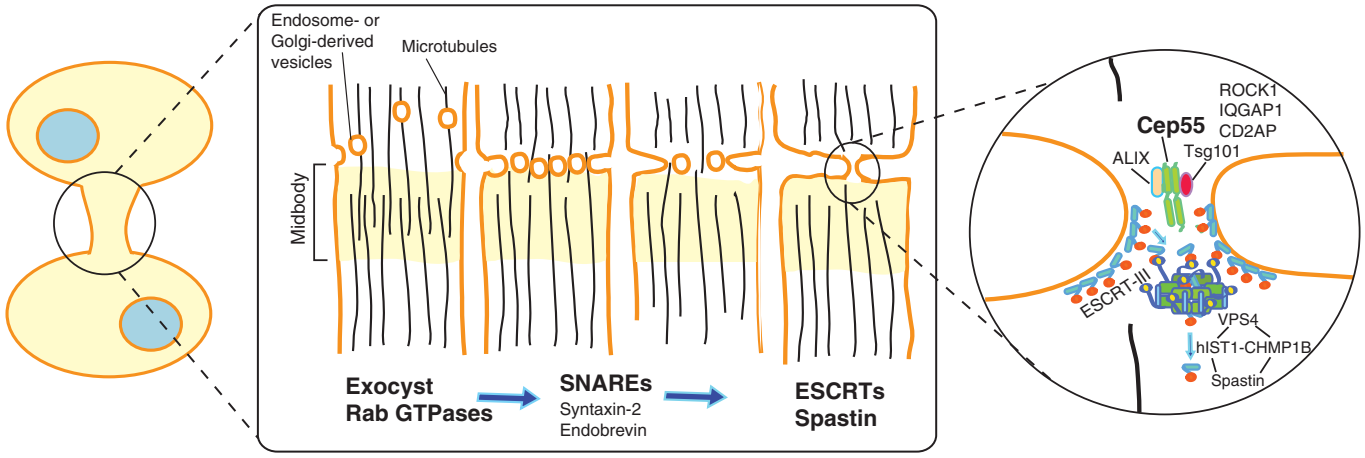

Fig. 3. A model for ESCRT-mediated abscission. Golgi- and endosome-derived vesicles are transported to the midbody along microtubules, a process that is regulated by Rab GTPases. Association with the exocyst-tethering complex at the midbody may constrain the site of vesicle fusion. The v-SNARE endobrevin (also known as VAMP8) and the plasma-membrane-associated t-SNARE syntaxin-2 mediate vesicle fusion with the plasma membrane. Cep55 recruits ALIX and Tsg101 to the midbody for subsequent ESCRT-III assembly. Tsg101 can interact with other proteins that are involved in cytokinesis – in particular CD2AP, ROCK1 and IQGAP. The coordinated recruitment of VPS4 and spastin, along with other regulatory proteins (hIST1 and CHMP1B), to the midbody may complete abscission by severing microtubules and mediating membrane fusion of the remaining tether via ESCRT-III disassembly (arrows).

cell localisation of these proteins occurs during cell division, and the expression of Vps4 mutants also caused phenotypes indicative of cell-division failure (Lindas et al., 2008; Samson et al., 2008). These data suggest that, in fact, the involvement of ESCRTs in cell division represents an ancestral role for these proteins, and the endosomal sorting function of ESCRTs in mammalian cells has evolved subsequently. This supports the hypothesis that ESCRT proteins play an essential role in abscission, which is independent of endosomal sorting. This idea is also supported by the phenotypes observed in hIST1-depleted cells, which are defective for abscission, although other ESCRT functions (such as the sorting of EGFR to MVBs and HIV-1 budding) are unaltered (Agromayor et al., 2009; Bajorek et al., 2009).

Midbody abscission by the ESCRT machinery presents a problem that needs to be resolved in terms of the diameter of the tether: the membranous tethers that restrain MVB vesicles and viral particles are thought to have a diameter in the region of 50-100 nm, whereas the midbody ring structure between two daughter cells is significantly larger, at around 1.5-2.0 μm (Gromley et al., 2005). However, there are several models that could explain the 'thinning' of the midbody to a point at which the ESCRT machinery could act directly. One possibility is that ESCRT proteins play a direct role in midbody thinning, as it has been suggested that membrane expulsion via budding vesicles may also be required to resolve the tether (Dubreuil et al., 2007). Alternatively, the fusion of secretory vesicles with the plasma membrane might give rise to a tether within the midbody on which the ESCRT machinery could act (Fig. 3). Interestingly, a role for spastin, a microtubule-severing enzyme, has also recently been implicated in ESCRT-mediated membrane scission. Spastin (60-kDa isoform) interacts with the ESCRT-III protein CHMP1B (Reid et al., 2005) and localises to the midbody in an MIT-domain- and CHMP1B-dependent manner. Depletion of spastin by siRNA, or the expression of CHMP1B non-binding mutants, causes cytokinetic failures, consistent with a role in abscission (Connell et al., 2009; Yang et al., 2008). One protein that could help coordinate the mechanical activities of VPS4 with those of spastin is hIst1, which binds to both ATPases (Agromayor et al., 2009). Both hIst1 and CHMP1 proteins are required to recruit VPS4 to the midbody (Bajorek et al., 2009). This phenotype could be explained by a mechanism in which the CHMP1B-hIst1 complex creates a network of MIMs that would increase the avidity for VPS4 and LIP5 at the midbody. Both CHMP1B and hIst1 also bind additional MIT-domain-containing proteins that might be required for abscission, including two deubiquitylating enzymes, AMSH and UBPY, and other proteins of unknown function (Agromayor et al., 2009). In fact, a role for UBPY and AMSH has been suggested (Mukai et al., 2008; Pohl and Jentsch, 2008), but more work is needed to determine whether the recruitment of these deubiquitylating enzymes by the ESCRT machinery is required for abscission.

In summary, the archaean ancestry and the association of ESCRT proteins with additional regulatory and enzymatic proteins suggests that the ESCRT machinery has a role in membrane scission, rather than an endosomal sorting function, at the midbody during cytokinesis. It is becoming clearer that the coordination of different activities such as microtubule severing, deubiquitylation and mechanical energy expenditure, with ESCRT function, is required to achieve abscission.

Concluding remarks and outstanding questions

Although we have made significant progress in recent years towards understanding ESCRT function, many questions remain. The emerging picture is one in which alternative adaptor proteins (such as Hrs, viral proteins and Cep55) recruit the core ESCRT machinery to resolve different membranous tethers by facilitating topologically equivalent membrane-scission events. In spite of recent progress in this area, the most pressing question of how exactly membrane scission is achieved is still unanswered. At present, we do not know whether the ESCRT proteins themselves mediate this membrane-scission event, or whether their role is to recruit additional proteins to do the job.

An additional layer of complexity in the ESCRT machinery that is poorly understood is the gene expansion in humans compared with yeast. For example, there are three isoforms of CHMP4 and two isoforms of VPS4 in humans, whereas yeast only have one form of each protein. More strikingly, the combination of one form of Tsg101, four isoforms of VPS37 (A, B, C and D), two of VPS28 (I and II) and two of MVB12 (A and B), gives a possible 16 different ESCRT-I complexes in humans. Thus, it will be important to determine whether different subsets of ESCRT proteins are preferentially required during endosomal sorting, retroviral replication and cytokinesis. Preliminary evidence that supports this idea includes functional studies that compared the different isoforms of CHMP4; these show that the overexpression of YFP-CHMP4C preferentially inhibits cytokinesis, whereas YFP-CHMP4B is the most potent inhibitor of HIV-1 release (Carlton et al., 2008). These results suggest that different isoforms of ESCRT-III might have evolved to mediate different scission events. However, more work is needed to confirm this hypothesis and to fully understand the functional differences within this and other genes that have been expanded in the human ESCRT machinery. It will also be interesting to determine the regulation and function of these multiple isoforms and to investigate the constellation of poorly characterised proteins that bind to ESCRT-III.

In conclusion, it is clear that, in addition to its role in viral infection, the ESCRT machinery is involved in several other cellular processes. Because malfunctions of these proteins have been implicated in diverse disease pathologies, developments in the understanding of ESCRT function could therefore provide important clues about the mechanisms of disease and lead to future therapeutic interventions.

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