

ESCRTs are everywhere

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Abstract

The ESCRT proteins are an ancient system that buds membranes and severs membrane necks from their inner face. Three "classical" functions of the ESCRTs have dominated research into these proteins since their discovery in 2001: the biogenesis of multivesicular bodies in endolysosomal sorting; the budding of HIV-1 and other viruses from the plasma membrane of infected cells; and the membrane abscission step in cytokinesis. The past few years have seen an explosion of novel functions: the biogenesis of microvesicles and exosomes; plasma membrane wound repair; neuron pruning; extraction of defective nuclear pore complexes; nuclear envelope reformation; plus-stranded RNA virus replication compartment formation; and micro- and macroautophagy. Most, and perhaps all, of the functions involve the conserved membrane-neck-directed activities of the ESCRTs, revealing a remarkably widespread role for this machinery through a broad swath of cell biology.

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Introduction

The endosomal sorting complex required for transport (ESCRT) complexes comprise an ancient system for membrane remodeling and scission. The ESCRTs were first discovered and named for their central role in sorting membrane proteins from endosomes to lysosomes (Katzmann et al, 2001). The formation of multivesicular bodies (MVBs) is a key step in this pathway, and the ESCRT proteins comprise the main machinery of MVB biogenesis (Hanson & Cashikar, 2012). MVBs are formed from endosomes by the inward budding of the limiting membrane into the lumen, followed by the severing of the narrow neck connecting the bud and the limiting membrane (Fig 1). Ubiquitinated membrane proteins are sorted into these buds by the ESCRT proteins. The severing event results in the release of cargo-laden intralumenal vesicles (ILVs) into the interior of the MVB.

The ESCRTs first emerged in the Archaea, and their earliest function was in cell division (Lindas et al, 2008; Samson et al, 2008). The Crenarchaeota orthologs of ESCRT-III subunits and the AAA+ ATPase Vps4 sever the narrow membrane neck connecting daughter cells, a role shared from the Crenarchaea to Schizosaccharomyces pombe to mammals (Samson & Bell, 2009) (Table 1). Thus, the primal biochemical function of the ESCRTs is to constrict and sever narrow membrane necks (although the biophysical mechanism of membrane scission remains unknown) (Hurley & Hanson, 2010; Peel et al, 2011; Henne et al, 2013; McCullough et al, 2013). The additional biochemical activities of the ESCRTs, such as membrane budding and the recruitment of ubiquitinated membrane proteins, are later elaborations (Samson & Bell, 2009; Wideman et al, 2014). These ubiquitin-associated activities are directed mainly by the ESCRT-I and ESCRT-II complexes and the ESCRT-associated protein ALIX. In yeast and animals, ubiquitinated cargo is organized into a flat clathrin-coated domain by ESCRT-0 prior to encountering ESCRT-I (Raiborg et al, 2001; Sachse et al, 2002). As an early endosomal clathrin adaptor and a later arrival in evolution (Wideman et al, 2014), ESCRT-0, in spite of its name, is not a core component of the membrane budding and scission machinery. The core machinery consists of ESCRT-I and ESCRT-II on the one hand, and ALIX on the other. ESCRT-I/ESCRT-II and ALIX function as two branches of the ESCRT pathway that feed into a common ESCRT-III and Vps4 membrane scission machinery (Fig 2). The structures of these proteins have been exhaustively studied and are well described elsewhere (Williams & Urbe, 2007; Hurley, 2010).

The modular organization of the ESCRT system and its versatility has made it an evolutionary success story. The initial discovery of ESCRT function in MVB biogenesis (Katzmann et al, 2001) was quickly followed by the realization that HIV and other viruses hijack the ESCRTs to bud from the plasma membrane of infected cells with the same membrane topology (Garrus et al, 2001; Martin-Serrano et al, 2001; VerPlank et al, 2001; Demirov et al, 2002) (Fig 3A). Six years later, this was followed by the discovery that the ESCRTs function in cell division in mammals (Carlton & Martin-Serrano, 2007) and in a subset of Archaea (Lindas et al, 2008; Samson et al, 2008) (Fig 4A). ESCRT-I and ESCRT-II are nearly as ancient as ESCRT-III and Vps4, as they are present in the genomes of the Lokiarchaeota, a proposed novel phylum of archaebacteria with eukaryote-like membrane trafficking processes (Spang et al, 2015).

It is deeply satisfying that all three of these pathways involve membrane neck severing with a common topology. These three cellular functions of the ESCRTs have been intensively studied and by now could be considered the "classical" functions of the ESCRTs. In the past several years, the classical repertoire of ESCRT functions has been surpassed by new roles. In this review, I take stock of the new additions to the list and place them in the context of the classical

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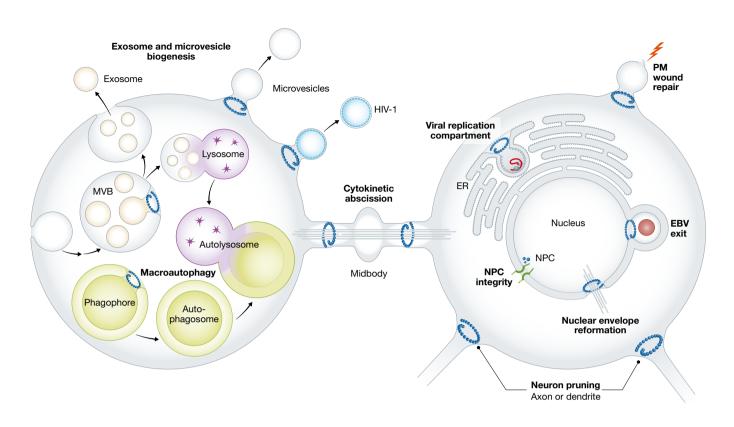


Figure 1. Overview of the cellular functions of the ESCRTs.

Table 1. Usage of ESCRT complexes in diverse cellular functions.

| | MVB | HIV-1 release | Cytokinesis | Exosome | Microvesicle | PM wound repair | Neuron pruning | EBV budding | NPC QC | NE reformation | VRRC |
|----------------------------------|---------------|------------------|-----------------|------------|--------------|------------------------|-------------------|----------------|-----------|-------------------|--------------------|
| Targeting element | ESCRT-0 Ub | Gag p6 | CEP55 | Syntenin | ARRDC1 Ub | ALG-2/Ca ²⁺ | ? | BFRF1 | Heh2 | UFD1 | TBSV-p33 BMV-1a |
| Upstream ESCRT | ESCRT-I | ESCRT-I ALIX | ESCRT-I ALIX | ALIX | ESCRT-I | ALIX | ESCRT-I | ALIX | None | None | ESCRT-I ALIX |
| Bridging ESCRT | ESCRT-II | ESCRT-II? | ESCRT-II? | Not needed | ? | Not needed | HD-PTP | Not needed | None | None | |
| Special ESCRT-III subunits | | | IST1 | | | | | | | CHMP7, IST1 | |
| Spastin | | | 1 | | | | ? | | | ✓ | |

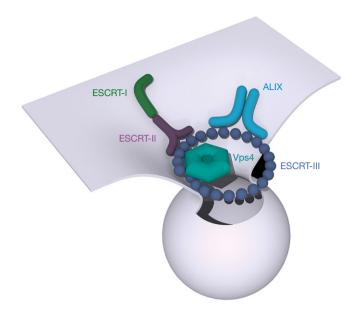
All of the pathways shown involve CHMP4/Snf7 and Vps4. Endosomal microautophagy mirrors the MVB pathway, and macroautophagy is not included due to insufficient data. The table emphasizes generalizations of typical situations that are mostly drawn from animal cells. Entries for nuclear pore complex quality control (NPC QC) and viral RNA replication compartment (VRRC) formation pertain to yeast. Requirements may vary in yeast, plants, Archaea, and in various special cases in animals. Plasma membrane budding of many animal viruses other than HIV-1 is also ESCRT dependent, but details of ESCRT usage vary.

functions. I consider to what extent the new functions fit with the established mechanistic paradigms and how they highlight both the strengths and deficiencies of current tools and concepts in the field.

Diagnosing the need for an ESCRT

Saccharomyces cerevisiae strains deleted in core ESCRT genes are viable. This makes Baker's yeast the most powerful model system for studying the classical role of ESCRTs in MVB biogenesis.

Dysfunction in MVB biogenesis in yeast is readily scored using model cargoes such as carboxypeptidase S (CPS). Other model organisms, such as *Drosophila melanogaster* (Thompson *et al*, 2005; Vaccari & Bilder, 2005; Herz *et al*, 2006) and *Arabidopsis thaliana* (Spitzer *et al*, 2006), are tractable for ESCRT genetics and have made important contributions to understanding the dependence or independence of various pathways on the ESCRTs. The loss of core ESCRT genes, such as *TSG101*, *VPS25*, and *CHMP5* which encode subunits of ESCRT-I, ESCRT-II, and ESCRT-III, respectively (see Table 2 for subunit compositions), is embryonically lethal in mice



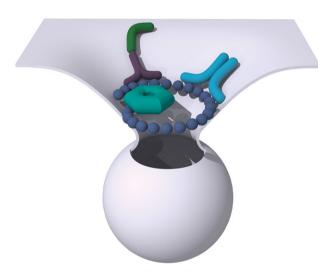


Figure 2. Two branches of the ESCRT pathway.

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The ESCRT-I/ESCRT-II and ALIX branches of the ESCRT pathway are illustrated in the context of a generic vesicle bud neck. The upstream ESCRTs (I/II and ALIX) are shown on the outer face of the bud neck with respect to ESCRT-III; however, the actual nanoscale arrangement of the molecules is unknown.

(Wagner *et al*, 2003; Shim *et al*, 2006; Handschuh *et al*, 2014). Mouse hypomorphs such as the ESCRT-II mutant *VPS25*^{ENU} are viable, but have striking developmental defects (Handschuh *et al*, 2014). Cells derived from hypomorphic mice promise to be useful in the future. CRISPR-Cas9-edited A549 cells genomically deleted in TSG101 are viable (Sanyal *et al*, 2013). Apart from this report, it is remarkable how few studies to date have used gene-edited mammalian cells in ESCRT research.

In the great majority of studies into ESCRT function in mammalian cells, RNA interference (RNAi) has been the tool of choice. Cultured cells have been shown to survive knockdown of even multiple core ESCRT genes (Morita *et al*, 2011). A loss of function upon ESCRT knockdown is the closest thing to a "gold standard" for the dependence of a mammalian cell process on the ESCRTs. The converse does not hold, however. The absence of a loss-of-function phenotype on knockdown is not necessarily robust evidence for the ESCRT independence of a pathway, given variability in the efficiency and duration of the loss of the expressed protein. In some cases, RNAi knockdowns have had no phenotype even when other lines of evidence have suggested an important role for ESCRTs. For example, knockdown of ESCRT-II subunits has no effect on HIV-1 release (Morita *et al*, 2011) and on mammalian cytokinesis (Morita *et al*, 2007). Biochemical reconstitution (Carlson & Hurley, 2012), imaging (Goliand *et al*, 2014), and dominant-negative studies (Goliand *et al*, 2014) support a functional role. In this instance, where negative findings from RNAi studies differ from those of other approaches, the field has yet to reach a consensus.

Of course, none of the foregoing experiments, on their own, differentiate between direct contributions to the step under study and indirect contributions through perturbations of established functions. Identification and knockdown of specific adaptors that distinguish unique aspects of ESCRT function are invaluable in this regard. In HIV-1 release, the p6 domain of Gag has this function (Garrus *et al*, 2001; Martin-Serrano *et al*, 2001; VerPlank *et al*, 2001; Demirov *et al*, 2002), and in mammalian cytokinesis, CEP55 has this role (Carlton & Martin-Serrano, 2007; Morita *et al*, 2007; Lee *et al*, 2008). Visualization of ESCRTs at the site of action is also an important criterion for a direct role.

There are no pharmacological inhibitors available for the ESCRT pathway, although overexpression of Walker EQ mutant VPS4 (Babst et al, 1998) is widely used and usually effective. Overexpression of an ALIX mutant defective in CHMP4 binding (Kim et al, 2005; Fisher et al, 2007; Usami et al, 2007) is a popular and effective tool for diagnosing a role for the ALIX-CHMP4 branch of the ESCRT pathway. Overexpression of a fragment of ESCRT-II subunit VPS25 involved in binding to ESCRT-III inhibits cytokinesis (Im et al, 2009; Goliand et al, 2014). Overexpression of GFP-tagged ESCRT-III subunits is also inhibitory, although this is typically considered something to be avoided in imaging studies, rather than actively pursued for functional inhibition. Biochemical reconstitution addresses what components are minimally sufficient rather than what is essential, but can be used to address what is essential in the context of a minimal system. Going forward, spatially (Loncle et al, 2015) or temporally (Matusek et al, 2014) restricted deletions of essential ESCRT genes will be helpful. In weighing the literature retrospectively, and in planning strategies to move ahead, it is important to bear these issues in mind.

ESCRTs, exosomes, and microvesicles

The terms exovesicle and extracellular vesicle refer to any biological vesicle extant outside of a cell, regardless of its origin (Raposo & Stoorvogel, 2013). Here I will use the term microvesicle to indicate extracellular vesicles that directly bud from the plasma membrane. "Microvesicle" is synonymous with "ectosome", "shedding vesicle", and "microparticle". In contrast, exosomes originate as ILVs within MVBs that fuse with the plasma membrane. MVBs are also referred to as multivesicular endosomes (MVEs).

The concept of an ESCRT role in exosome biogenesis is not new and seems natural given that exosomes originate in MVBs and that

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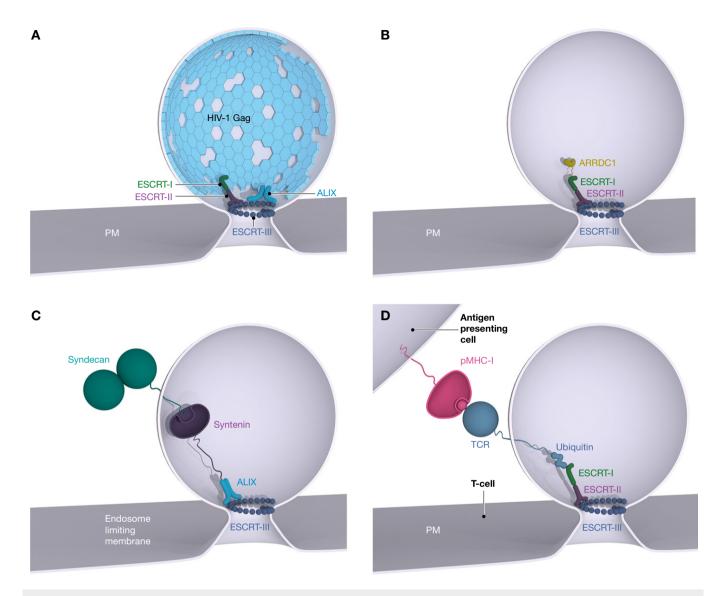


Figure 3. Viral and vesicular budding by the ESCRTs.

(A) Budding of HIV-1 from the plasma membrane of an infected human cell uses both ESCRT-I and ALIX. ESCRT-II is shown despite a lack of genetic evidence for its role in HIV-1 budding, because it is capable of bridging ESCRT-I and ESCRT-III in vitro, and a bridging factor of some kind is necessary. (B) ARRDC1 mediates microvesicle budding through the ESCRT-I pathway. (C) Syntenin mediates ALIX recruitment in the biogenesis of syndecan-containing exosomes. (D) T-cell receptor-containing microvesicles are shed into the immunological synapse in an ESCRT-I-dependent process. Vps4 is required in all of these processes but not shown.

ESCRTs comprise the major machinery for MVB biogenesis. However, several prominent studies employing dominant-negative VPS4 (Trajkovic *et al*, 2008) and knockdowns (van Niel *et al*, 2011) reported negative or mixed findings with respect to ESCRT requirements in exosome biogenesis. The tetraspanin CD63 was consistently observed in exosomes, suggesting that tetraspanins might comprise a separate mechanism for the biogenesis of MVBs destined for fusion with the plasma membrane and exosome release (van Niel *et al*, 2011). Evidence began to emerge in 2011–2012 that ESCRTs play a direct role in exovesicle biogenesis. The first suggestion came from the observation that a peptide from the SP2 region of HIV-1 Gag inhibited both exovesicle release and VPS4B association with exovesicle cargoes (Gan & Gould, 2011). In *C. elegans*, loss of the lipid flippase TAT-5 leads to a buildup of phosphatidylethanolamine

on the outside of the plasma membrane, which triggers extensive microvesicle shedding (Wehman *et al*, 2011). Increased shedding correlates with increased ESCRT localization at the PM, which is further increased by depletion of Vps4. RNAi against ESCRT-0 and ESCRT-I subunits partially suppresses shedding, but depletion of the key ESCRT-II and ESCRT-III subunits has no effect, leaving a mixed picture (Wehman *et al*, 2011). In a contemporaneous effort, the ubiquitin ligase adaptor ARRDC1 was shown to directly recruit ESCRT-I to microvesicle budding sites via its PSAP motif (Fig 3B). The TSG101 knockdown, VPS4-DN expression, and PSAP mutant were shown to have robust phenotypes (Nabhan *et al*, 2012), strengthening the case for an ESCRT-microvesicle connection.

Syndecans are the major heparan sulfate-presenting proteins on the cell surface, and they are also found in exosomes. Syndecan-positive

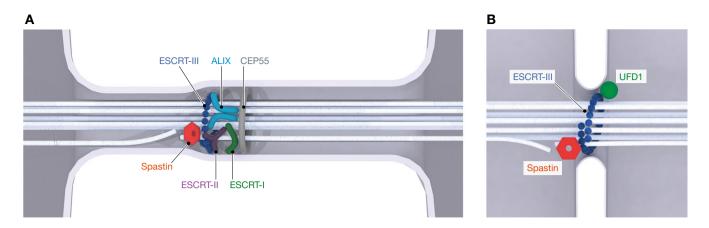


Figure 4. Membrane neck scission by ESCRTs in cell division.

(A) The classical cytokinetic function of both the ESCRT-I/ESCRT-II and ALIX branches of the pathway in membrane abscission, and coordination with microtubule severing by spastin. (B) Resolution of gaps between fragments of reforming nuclear membrane in telophase, again coordinated with microtubule severing. Vps4 is required in both pathways but not shown.

Table 2. Composition of the ESCRT complexes.

| Yeast protein | Alternative names (yeast) | Metazoan protein | Alternative names (metazoan) | Motifs and domains |
|------------------|--|--|---|--|
| Vps23 | Stp22 | TSG101 | | UEV, PRD, stalk, headpiece |
| Vps28 | | VPS28 | | Headpiece, Vps28 CTD |
| Vps37 | Srn2 | VPS37A, B, C, D | | Basic helix, stalk, headpiece |
| Mvb12 | • | MVB12A, B, UBAP1 | | Stalk, MAPB |
| Vps22 | Snf8 | EAP30 | | Basic helix, Winged-helix |
| Vps25 | | EAP20 | | Winged-helix |
| Vps36 | | EAP45 | | Winged-helix, GLUE, NZF1 (yeast), NZF2 (yeast) |
| Vps2 | Did4 Chm2 | СНМР2А, В | | MIM1 |
| Vps20 | Chm6 | CHMP6 | | MIM2 |
| Vps24 | | CHMP3 | | Weak MIM1 |
| Snf7 | Vps32 | CHMP4A, B, C | Shrub | Weak MIM2 |
| Vps60 | Chm5 | CHMP5 | | |
| Did2 | Chm1 Vps46 | СНМР1А, В | | MIM1 |
| | | CHMP7 | | |
| lst1 | | IST1 | | MIM1, MIM2 |
| Vps4 | | VPS4A, B | SKD1 | MIT, AAA |
| Vta1 | | VTA1 | LIP5 | MIT |
| Bro1 | Vps31 | ALIX | AIP1 | Bro1, V, PRD |
| | Protein Vps23 Vps28 Vps37 Mvb12 Vps22 Vps25 Vps36 Vps20 Vps24 Snf7 Vps60 Did2 Ist1 Vps4 Vta1 Vps4 Vta1 | Yeast protein names (yeast) Vps23 Stp22 Vps28 Vps37 Vps37 Srn2 Mvb12 Vps22 Vps25 Vps36 Vps2 Did4 Chm2 Vps20 Chm6 Vps24 Snf7 Vps32 Vps60 Chm5 Did2 Chm1 Vps46 Ist1 Vps4 Vta1 Vta1 | Yeast protein names (yeast) Metazoan protein Vps23 Stp22 TSG101 Vps28 VPS28 VPS28 Vps37 Srn2 VPS37A, B, C, D Mvb12 MVB12A, B, UBAP1 Vps22 Snf8 EAP30 Vps25 EAP20 Vps36 EAP45 Vps2 Did4 Chm2 Vps20 Chm6 CHMP6 Vps24 CHMP3 Snf7 Vps32 CHMP4A, B, C Vps60 Chm5 CHMP5 Did2 Chm1 CHMP1A, B Vps46 CHMP7 Ist1 IST1 Vps4 VPS4A, B Vta1 VTA1 | Yeast protein names (yeast) Metazoan protein names (metazoan) Vps23 Stp22 TSG101 Vps28 VPS28 Vps37 Srn2 VPS37A, B, C, D Mvb12 MVB12A, B, UBAP1 Vps22 Snf8 EAP30 Vps25 EAP20 Vps36 EAP45 Vps2 Did4 Chm2 Vps20 Chm6 Vps24 CHMP6 Vps24 CHMP3 Snf7 Vps32 CHMP4A, B, C Shrub Vps60 Chm5 CHMP5 Did2 Chm1 CHMP1A, B CHMP1A, B Vps46 CHMP7 Ist1 IST1 Vps4 VPS4A, B SKD1 Vta1 VTA1 LIP5 |

UEV, ubiquitin E2 variant, a ubiquitin-binding domain with no catalytic activity; PRD, proline-rich domain; CTD, C-terminal domain; MAPB, MVB12-associated β-prism domain; GLUE, GRAM-like ubiquitin binding in EAP45, a variant PH domain that also binds to PI(3)P; NZF, neural zinc finger domain; MIM, MIT-interacting motif; MIT, microtubule interacting and trafficking; AAA, ATPases associated with various cellular activities; Bro1, N-terminal CHMP4-binding domain of Bro1, ALIX, and related proteins; V, V-shaped ubiquitin and signal binding central domain of Bro1 and ALIX.

exosomes contain CD63 and ceramide and thus qualify as "canonical" exosomes. Syndecans are sorted into exosomes by a specific adaptor protein, syntenin. Syntenin uses a YPXL motif to bind

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specifically to ALIX (Baietti *et al*, 2012; Hurley & Odorizzi, 2012) (Fig 3C). Downstream of ALIX, CHMP4, and VPS4 knockdowns block syndecan exosome biogenesis (Baietti *et al*, 2012). Knockdown

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of CD63 does not block the biogenesis of these exosomes, but the ESCRT knockdowns were shown to decrease the amount of CD63 released (Baietti *et al*, 2012). This portrays CD63 as a fellow traveler with syndecan rather than a core component of the exosome release pathway. The Cos transmembrane proteins of yeast have been proposed to serve as the yeast counterpart of mammalian tetraspanins (MacDonald *et al*, 2015). Ubiquitinated Cos proteins provide a sorting signal in trans to non-ubiquitinated cargo destined for ILVs and so facilitate their sorting by the ESCRTs (MacDonald *et al*, 2015). The concept that ESCRTs and CD63 could work together by similar means is attractive.

The use of temporally controllable RNAi targeted to Hedgehog (Hh)-producing cells made it possible to explore the role of ESCRTs in long range Hh signaling in wing development in Drosophila (Matusek et al, 2014). Knockdown of ALIX and subunits of ESCRT-I, ESCRT-II, and ESCRT-III block the Hh signal (Matusek et al, 2014). Hh is secreted from cells in exovesicles that contain ~160 other proteins, including several of the ESCRTs (Matusek et al, 2014). These exovesicles appear to be shed directly from the plasma membrane. At the immunological synapse, T-cell receptors (TCR) form complexes with pathogen-bound major histocompatibility complexes (pMHC) on antigen-presenting cells. These complexes are immobilized when the TCR-containing microvesicles are secreted into the synaptic center. This process was convincingly shown to be dependent on ESCRT-I and VPS4 (Choudhuri et al, 2014) using RNAi and dominant-negative approaches. The process is closely analogous to the release of HIV-1 virions from the plasma membrane of infected T cells (Fig 3D), and it is natural to infer that HIV-1 co-opted its use of the ESCRT from the TCR microvesicle budding mechanism (Choudhuri et al, 2014). To sum up, the sustained findings in the past few years of multiple examples of ESCRT-dependent exosome and microvesicle biogenesis in diverse species and contexts suggest that the ESCRTs have a ubiquitous and fundamental role in these processes.

ESCRTs and plasma membrane wound repair

When eukaryotic cells are perforated in their plasma membranes, they react to patch the holes within a matter of seconds (Andrews *et al*, 2014). The increase in [Ca²⁺] at the site of the wound signals the need for repair. Holes can be created experimentally by detergents, pore-forming toxins, mechanical injury, or a laser. In every one of these cases, ALIX, ESCRT-III, and VPS4 are recruited subsequent to the Ca²⁺ signal (Jimenez *et al*, 2014). The central ESCRT-III subunit CHMP4B is particularly important, and cell survival following wounding is compromised by its knockdown. The damaged membrane is shed outward from the plasma membrane in much the same manner as in microvesicle budding (Jimenez *et al*, 2014).

The initial recruitment of ESCRTs to wound sites is rapid and energy independent and thus does not require ubiquitination (Jimenez *et al*, 2014). Consistent with the initial ubiquitin independence of the pathway, and the key role of Ca²⁺, the ESCRTs are recruited via the EF hand protein ALG-2 (Scheffer *et al*, 2014). In its Ca²⁺-bound state, ALG-2 directly interacts with ALIX. ALG-2 appears to be the unique ESCRT-recruitment element in plasma membrane wound repair (Scheffer *et al*, 2014), analogous to CEP55 in cytokinesis, the Gag p6 domain in HIV-1 budding, or the syntenin YPXL motif in syndecan exosome biogenesis (Table 1).

ESCRTs and neuron pruning

Early in their development, neurons generate a profusion of branches. These branches, both dendrites and axons, are subject to shortening. Extending the branch metaphor, this process is referred to as "pruning". Drosophila has been a model system of choice for the study of neuron pruning. Three different laboratories have now shown that the ESCRT machinery is deeply involved in this process in Drosophila. In the first of these studies to appear, RNAi against ESCRT-I (Vps28) and ESCRT-III (Shrub, the Drosophila CHMP4 ortholog) and dominant-negative Vps4 blocked dendrite pruning. The effect was attributed to a block in the downregulation of the cell surface protein neuroglian (Nrg). It was postulated the Nrg inhibits dendrite pruning, which is relieved when Nrg is endocytosed and downregulated via the MVB pathway and lysosomal degradation (Zhang et al, 2014). This was followed by a report that ESCRT-0 is required for axon pruning (Issman-Zecharya & Schuldiner, 2014). In this case, the finding is that another cell surface receptor, Patched (Ptc), is not downregulated normally when ESCRT function is lost, leading to sustained inhibition of pruning. To the extent that the receptor downregulation mediates this effect, this is not a "new" function of the ESCRTs, rather a new instance of the ESCRTs' classical role in the MVB pathway.

The putative roles of Nrg and Ptc as pruning inhibitors beg the question as to the molecular mechanism of pruning itself. A third study suggested that the ESCRTs are directly involved in severing the membrane necks of both axons and dendrites (Loncle et al, 2015). This function of the ESCRTs requires ESCRT-I and ESCRT-III, but not ESCRT-II or ALIX. In this instance, the ESCRT-II independence is persuasive, as homozygous deletion of vps25 in the context of mosaic analysis with a repressible cell marker (MARCM) recapitulates the RNAi phenotype. It is unusual for an ESCRT-III-dependent process to require neither ESCRT-II nor ALIX. ALIX binds to CHMP4 through its Bro1 domain; however, several other Bro1-domaincontaining proteins exist in animals. One of these is HD-PTP, which is required for neuron pruning and whose function requires an intact CHMP4-binding Bro1 domain. In this study, it was possible to partially separate the roles of the ESCRTs in MVB biogenesis from their specialized role in neuron pruning on the basis of the differential requirement for ESCRT-II. By doing so, an essential non-MVB role for ESCRTs was demonstrated. Imaging of ESCRT localization shows concentrations of ESCRT-III at sites in dendrites that go on to become sites of severing. At present, it is unclear what upstream signal recruits and activates the ESCRTs at scission sites. It is also unclear whether Ptc or Nrg is involved in negatively regulating this aspect of ESCRT function, which if so would represent a double-negative feedback loop. What is clear is that membrane scission of dendrites and axons represents another important addition to the list of genuinely new functions for the ESCRTs (Loncle et al, 2015).

ESCRTs and the nucleus

In 2001, two back-to-back papers from Hollenberg and co-workers identified CHMP1, respectively, as both a cytoplasmic vesicle trafficking protein (Howard *et al*, 2001) and a nuclear matrix protein (Stauffer *et al*, 2001). Given its ambiguous nature, it was

named chromatin modifying protein/charged multivesicular body protein-1. In the subsequent decade, the cytosolic membrane-remodeling functions of CHMP1 and many other CHMPs were thoroughly explored. While no evidence has emerged for a putative chromatin remodeling function for CHMP1, other functions of CHMP1 in the nucleus have come to the fore. Evidence for nuclear roles for the ESCRTs began to emerge in 2012, with virological studies leading the way. Epstein–Barr virus (EBV) assembles in the nucleus and is too large for export through the nuclear pore complex (NPC). Instead, EBV exits the nucleus by budding through the nuclear envelope. ESCRTs were shown to be required for the EBV budding through the nuclear membrane (Lee *et al*, 2012). The BFRF1 protein of EBV recruits ALIX, which in turn recruits CHMP4B.

It took until 2014 for physiological (as opposed to pathophysiological) functions to emerge for nuclear ESCRTs. In budding yeast, the nuclear envelope does not break down during the cell cycle. NPCs are long-lived and their quality control is important. ESCRT-III subunits and Vps4 were identified as factors important for NPC integrity in an epistatic screen in yeast (Webster et al, 2014). None of the upstream ESCRTs are involved in the pathway. Rather, the nuclear inner membrane protein and NPC quality control factor Heh2 appear to directly recruit ESCRT-III. The molecular mechanism for the detection and removal of flawed NPCs remains uncertain. One attractive possibility is that Vps4 might extract subunits of misassembled NPCs. Indeed, Vps4 is a disassembly machine that completely unfolds its substrates (Yang et al, 2015), like other AAA+ ATPases involved in disaggregation and degradation. In this scenario, ESCRT-III would serve as an adaptor for Vps4 rather than as a membrane-remodeling machine.

Finally, 2015 brought compelling evidence that nuclear ESCRTs have a physiological role in membrane remodeling. In animals, the nuclear envelope breaks down and must be reformed in every round of cell division. The mechanism by which nuclear envelope fragments are resealed has been unknown. Resealing entails the same changes in membrane topology that have become familiar from other ESCRT reactions. Resealing occurs during telophase. Armed with the hypothesis that ESCRTs might be involved, two groups imaged ESCRT-III localization in telophase (Olmos et al, 2015; Vietri et al, 2015). As with NPC quality control, upstream ESCRT proteins do not appear to be required. Rather, UFD1, a subunit of the p97 complex, seems able to directly recruit ESCRT-III to sites of sealing (Olmos et al, 2015). The "orphan" ESCRT-III protein CHMP7 is required for the process, giving it a job to do for the first time (Vietri et al, 2015). Nuclear envelope formation requires the severing of spindle microtubules that would otherwise physically obstruct membrane sealing (Fig 4B). A similar problem needs to be solved in cytokinesis, where the central spindle must be severed before the membrane neck surrounding it can be sealed. The two ESCRT-III subunits CHMP1B and IST1 bind with high affinity to the microtubule-severing enzyme spastin (Yang et al, 2008; Yu et al, 2008; Connell et al, 2009). In cytokinesis, CHMP1B recruits the microtubule-severing enzyme spastin such that microtubule and membrane neck severing are coordinated at the same site by an elegant mechanism (Yang et al, 2008). In the case of nuclear envelope reformation, CHMP7 recruits IST1 to the same end (Vietri et al, 2015). The parallels to the mechanism of membrane abscission in cytokinesis, and the consistency with the biochemistry and biophysics of these proteins, are truly satisfying. The reconciliation of the apparently separate functions of ESCRTs in membrane remodeling and in the nucleus is equally satisfying.

ESCRTs and viral replication compartments

Plus-stranded RNA viruses such as the tombusviruses and bromoviruses of plants replicate in compartments that are protected by a limiting membrane. Tomato bushy stunt virus (TBSV), which can also replicate in yeast, uses its p33 protein to recruit ESCRT-I and Bro1 (yeast ALIX), and in turn, ESCRT-III (Barajas et al, 2009). The ESCRTs are involved in the budding of the viral replication complex into a membrane-delimited vesicular compartment. The bromovirus brome mosaic virus (BMV) also uses ESCRT-III to build itself a protected replication compartment (Diaz et al, 2015). The 1a protein of BMV recruits the ESCRT-III protein Snf7 directly (Diaz et al, 2015), without the apparent need for upstream factors. In contrast to the situation with retroviral budding from the plasma membrane, these budded replication compartment are not actually severed from the limiting membrane. Since they use much of the same machinery, what prevents membrane scission here is an interesting question.

ESCRTs and autophagy

The term autophagy refers to any of several cellular self-consumptive processes, macroautophagy, microautophagy, and chaperonemediated autophagy. These processes are essential both for cell survival during starvation and for the clearance of a wide variety of unnecessary or harmful materials from the cell. In microautophagy, cytosolic materials are directly taken up into the lysosome. In the endosomal version of this, material is taken up in intralumenal vesicles of MVBs, which subsequently fuse with the lysosome. Since the ESCRTs are so central to ILV formation and MVB biogenesis, it perhaps should not be surprising that they are also required for endosomal microautophagy (Sahu et al, 2011). The chaperone Hsc70 appears to act as an adaptor to select certain cytosolic protein and recruit them to the late endosomal limiting membrane for internalization by ESCRTs (Sahu et al, 2011). This role for the ESCRTs could be considered a new use of their classical function in MVB biogenesis.

The term "autophagy" is often used as a synonym for "macroautophagy", the uptake of bulk cytosol or large subcellular structures by a double-membrane phagophore that grows and engulfs its substrates. "Macroautophagy" will be used here since this article also concerns microautophagy. It has been known for some time that the ESCRTs are required for macroautophagy (Lee et al, 2007; Rusten et al, 2007). It is becoming clear that the ESCRT pathway is upregulated in advance of and in coordination with the larger macroautophagic response to starvation (Jones et al, 2012; Mueller et al, 2015). What has been much less certain is whether the ESCRTs have a direct role in the remodeling of the phagophore membrane as it grows and closes around its cargo. In principle, the macroautophagic phenotypes of ESCRT mutants, as reported through 2014, could be accounted for by their essential but indirect roles in the MVB pathway (Filimonenko et al, 2007). This has remained the prevailing model in the field, in part because it is

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essentially impossible to distinguish closed autophagosomes from nearly closed ones by electron microscopy. In mammals, incorporation of the SNARE protein syntaxin-17 into autophagosomes is important for their fusion with lysosomes, and syntaxin-17 incorporation seems to depend upon their closure (Itakura *et al*, 2012). Thus, failure to close late in autophagosome biogenesis would presumably appear as a defect in fusion with the lysosome.

The prevailing view notwithstanding, the topology of the closure of the phagophore double membrane should make it, in theory, an ideal substrate for sealing by the ESCRTs. Now, incisive and exciting imaging studies of chmp1 Arabidopsis show large and abundant unclosed phagophores (Spitzer et al, 2015). Ultimately, autophagic cargo does reach its destination in chmp1 cells, but the process is substantially delayed. If these images are the smoking gun, the eyewitness account of the crime has yet to be produced: direct imaging of ESCRTs localized at shrinking rim of the autophagic cup at the moment before closure. This unique and transient event may be extraordinarily difficult to capture. In the absence of this final piece of evidence, the preponderance of the data now favor a direct role in phagophore closure, at least for ESCRT-III, and at least for plastid autophagy in plants. Given the conservation of the ESCRT system and its function, though, it would be surprising if this role were not more widespread.

Concluding remarks

The last few years has seen in quiet revolution in ESCRT cell biology. The new functions discovered or confirmed outnumber the classical function several times over. It is now hard to think of any example of "reverse" topology membrane budding and scission in eukaryotes (or in some Archaea) that does not involve the ESCRTs. Many of the most interesting discoveries are being made by cell and developmental biologists who are new to the ESCRT field—more evidence that the ESCRTs have gone mainstream in cell biology. It is hard to know how much farther the field can go in finding new functions. The frontier questions now may not be so much what do the ESCRTs do or why, but rather how they do what they do.

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Conflict of interest

The author declares that he has no conflict of interest.

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