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The ESCRT machinery: new functions in viral and cellular biology

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Abstract

The ESCRT (endosomal sorting complex required for transport) machinery consists of a number of cytosolic proteins that make up three functional subcomplexes: ESCRT-I, ESCRT-II and ESCRT-III. These proteins function in multivesicular body formation and cell division and are co-opted by enveloped retroviruses to facilitate viral egress. Analysis of these functions may help illuminate conserved mechanisms of ESCRT function.

The ESCRT (endosomal sorting complex required for transport) machinery

The ESCRT machinery comprises the protein products of the Class E subset of Vps (vacuolar protein sorting) genes, identified as being required for sorting biosynthetic cargo into the yeast vacuole. A wealth of genetic and biochemical evidence has been documented to support a role for this machinery in the biogenesis and sorting of cargo on to intraluminal vesicles of MVBs (multivesicular bodies), a particular class of late endosome [1,2]. The ESCRT machinery consists of three multisubunit protein complexes, ESCRT-I, ESCRT-II and ESCRT-III, each comprising a number of individual ESCRT subunits (Table 1). In addition to the major complexes, a variety of ESCRT-associated proteins are recruited to this machinery through various interaction motifs and act to regulate ESCRT function. Detailed structural information has been generated for many constituents of these complexes and has led to the proposal that the ESCRT machinery forms a lattice on the endosomal surface [3–5], from where it can drive MVB biogenesis and/or cargo sorting on to nascent intraluminal vesicles. In the present article, we aim to review new data regarding functions of the ESCRT machinery in cellular and viral biology.

Hijacking ESCRTs

In addition to regulating endosomal sorting, the ESCRT machinery participates in the life cycle of enveloped retroviruses [6–8]. Retroviruses such as HIV-1, EIAV (equine infectious anaemia virus) and MLV (murine leukaemia virus) encode short sequence motifs called L-domains (late domains) within their structural (Gag) polyproteins that are essential for the release of assembled viruses from the host cell. When L-domain activity is compromised, viral release is arrested at a late stage, with nascent virions tethered to the cellular plasma membrane by thin membranous stalks [9]. Cellular partners for these L-domains have been identified as ESCRT components: P(S/T)AP L-domains bind to the UEV (ubiquitin E2 variant) domain of Tsg101 (tumour susceptibility gene 101) [10–13], LYPXL L-domains are able to co-ordinate the V-domain of Alix [ALG-2 (apoptosis-linked gene 2)-interacting protein X] [14,15] and PPXY L-domains recruit the ESCRT machinery through binding to the Nedd4 (neural-precursor-cell-expressed developmentally down-regulated 4)-family of ubiquitin ligases [16–19]. Disruption of ESCRT function, e.g. by RNA interference or expression of dominant-negative Vps4 [11–14], arrests viral release at a late stage, mimicking the effects of L-domain abolition and demonstrating clearly that the ESCRT machinery regulates viral release from the plasma membrane.

Mutant HIV-1 exposes alternative exit mechanisms

It has become evident that retroviruses can employ both primary and secondary L-domains to achieve their egress; for example, HIV-1 primarily uses a Tsg101-binding PTAP L-domain within p6 Gag (Figure 1); however, p6 Gag harbours secondary L-domain sequences [14,20], and, although HIV-1 release is largely dependent upon its PTAP L-domain, when this is disrupted [for example, by mutation of the PTAP sequence (HIV-1 PTAP$^-$)], particle release becomes dependent on other factors, one of which is its auxiliary Alix-binding LYPXL L-domain [14,21,22] (Figure 1). Although these L-domains differ in relative importance, their retention and conservation indicates that auxiliary L-domains confer an evolutionary advantage, perhaps allowing productive infection of a broad range of cells with a variety of ESCRT expression levels.

Under most conditions, HIV-1 release through its auxiliary L-domain is inefficient; however, this can be enhanced via overexpression of Alix. Two recent reports [21,22] have highlighted an essential role for Alix's C-terminus in regulating this release, suggesting the requirement for an as yet

Key words: endosomal sorting complex required for transport (ESCRT), multivesicular body (MVB), retrovirus, tumour susceptibility gene 101 (Tsg101), vacuolar protein sorting 4 (Vps4), virus.

Abbreviations used: Alix, ALG-2 (apoptosis-linked gene 2)-interacting protein X; CA, capsid; Cep55, centrosome protein 55; Chmp, charged multivesicular body protein 4; EIAV, equine infectious anaemia virus; ESCRT, endosomal sorting complex required for transport; L-domain, late domain; MVB, multivesicular body; NC, nucleocapsid; Nedd4, neural-precursor-cell-expressed developmentally down-regulated 4; Tsg101, tumour susceptibility gene 101; UBD, ubiquitin-binding domain; Vps, vacuolar protein sorting; YFP, yellow fluorescent protein.

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Table 1 | **ESCRT proteins in yeast and humans**

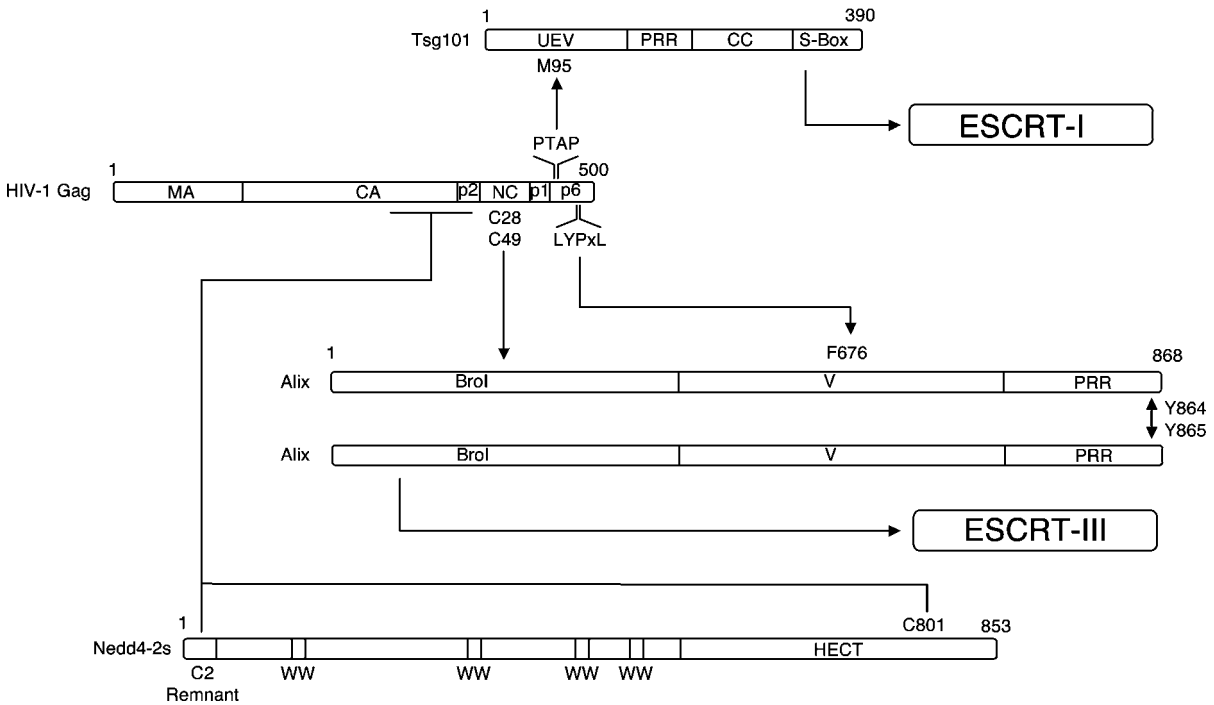
Protein complex	Yeast	Mammalian
ESCRT-I	Vps23p	Tsg101
	Vps28p	Vps28
	Vps37p	Vps37a, Vps37b, Vps37c, Vps37d
	Mvb12p	Mvb12a, Mvb12b
ESCRT-II	Vps22p	Eap30
	Vps25p	Eap20
	Vps36p	Eap45
ESCRT-III	Did2p	Chmp1a, Chmp1b
	Vps2p	Chmp2a, Chmp2b
	Vps24p	Chmp3
	Snf7p	Chmp4a, Chmp4b, Chmp4c
	Vps60p	Chmp5
	Vps20p	Chmp6
	?	Chmp7
Accessory	Bro1p	Alix
	Doa4p	UBPY, AMSH
	Vps4p	Vps4a, Vps4b
	Vta1p	Lip5
	Ist1p	Ist1

unidentified binding partner for this region. More recently, it has been shown that Alix's extreme C-terminus allows it to bind to itself and that Alix's ability to multimerize correlates well with its ability to support HIV-1 release [23]. Indeed, forcing Alix multimerization by appending a heterologous multimerization domain on to a C-terminally deleted version of Alix restores the ability of this protein to support HIV-1 release [23]. It is unclear why Alix needs to multimerize to allow rescue of HIV-1 release; one possibility is that this allows the adoption of a defined structure that is important for ESCRT activity. Alternatively, multimerization may simply act to enhance the interaction between Alix and HIV-1 Gag through avidity effects, much as is observed in a variety of biological systems [24]. It is known that, whereas isolated L-domain peptides from both HIV-1 p6 and EIAV p9 bind the same site within Alix's V-domain with comparable affinities [25], Alix actually binds EIAV p9 50–60-fold more strongly that it binds HIV-1 p6 [21,26], indicating a context-dependence to the interaction of Alix with L-domains and suggesting that multimerization may well strengthen the weak interaction of Alix with HIV-1 p6. Supporting this theory, deletion of Alix's C-terminus disrupts the Alix/HIV-1 p6 interaction, but leaves binding to EIAV p9 intact [27], and a multimerization-defective version of Alix is able to support release of an L-domain-defective version of HIV-1 complemented *in trans* with the p9 L-domain from EIAV [23]. These data suggest that the role of the extreme C-terminus of Alix may be to strengthen the interaction between Alix and HIV-1 p6 through multimerization-mediated enhancement of avidity.

However, it is not just Alix overexpression that can rescue the release of HIV-1 PTAP$^-$. Two recent reports detail that overexpression of a specific isoform of a Nedd4 E3 ubiquitin ligase (Nedd4-2s/Nedd4L, hereafter referred to as Nedd4-2s) can also enhance release of HIV-1 lacking L-domains [28,29]. Nedd4-2s is a naturally expressed truncated form of Nedd4-2 lacking most of its N-terminal C2 domain (Figure 1). Quite how this ligase connects these compromised HIV-1 viruses with the ESCRT machinery is unknown; HIV-1 Gag lacks PPXY L-domains typically used to recruit Nedd4 family ubiquitin ligases, and the ability of Nedd4-2s to rescue particle release was independent of its WW domains, canonical PPXY L-domain-binding modules. Utilization of minimal Gag constructs demonstrated that MA (matrix), the N-terminus of CA (capsid), NC (nucleocapsid) or p6 did not participate in the rescue of these crippled HIV-1, indicating that C-terminal regions of HIV-1 CA and SP2 may harbour *cis*-acting activities that can rescue release of HIV-1 PTAP$^-$ and may even form a binding site for Nedd4-2s [28,29]. Interestingly, the stimulatory effect of Nedd4-2s upon HIV-1 release was annulled by Tsg101 suppression, showing that when utilizing Nedd4-2s to access the ESCRT machinery, HIV-1 still acts through ESCRT-I [29]. In contrast, Tsg101 suppression did not affect the rescue of HIV-1 PTAP$^-$ release achieved through Alix overexpression [29], indicating that L-domain-compromised viruses can access the ESCRT machinery by multiple mechanisms. Additionally, mutation of a single cysteine residue within Nedd4-2s's active site prevented both its incorporation into virions and its ability to restore release of PTAP$^-$ HIV-1 [28,29]. The catalytic activity of Nedd4-2s's HECT (homologous with E6-associated protein C-terminus) domain and the ability of this protein to rescue HIV-1 PTAP$^-$ release also required residues at its extreme N-terminus that represent remnant sequences from the truncated C2 domain (Figure 1), suggesting that Nedd4-2s's catalytic activity may be subject to autoregulation [28]. These observations demonstrate that ubiquitin transfer is important for rescue of HIV-1 PTAP$^-$ release, although the target for this transfer is unknown. Ubiquitination plays a complex role in retroviral budding [8]. On the one hand, it can directly stimulate viral release, e.g. mutation of ubiquitin-acceptor sites in HIV-1 and rous sarcoma virus Gag induces late defects [30,31] and ubiquitin fusion to an L-domain-defective EIAV Gag could restore ESCRT-dependent release of this protein [32]. Countering this, Bieniasz and colleagues have demonstrated L-domain-dependent release of a prototypic foamy virus Gag engineered to contain no ubiquitin-acceptor sites [33]. Furthermore, when release of this virus was rendered dependent on PPXY L-domains, ubiquitin-ligase activity was still required for viral release, suggesting that ubiquitin transfer on to a downstream adaptor protein is critical for this activity [16,33]. In any case, Nedd4-2s can ubiquitinate HIV-1 Gag, suggesting that this may allow its recognition by UBDs (ubiquitin-binding domains) within the ESCRT machinery and it has even been suggested that ubiquitin itself attached to Nedd4-2s's catalytic site may serve as an interaction site for UBDs [29]. Alternatively, ubiquitination of the ESCRT machinery itself, or an as yet undefined cofactor, may be responsible for mediating this

Figure 1 | **Mechanisms employed by HIV-1 to exit from cells**

Diagram depicting interactions made by various regions of HIV-1 Gag with cellular proteins to effect viral exit. Known interactions represented by arrows, and putative interactions are shown by lines. MA, matrix. PRR, proline-rich region; UEV, ubiquitin 2 variant.



release. Whatever the case, the link between HIV-1, Nedd4-2s and the ESCRT machinery is not yet fully defined and future developments involving these crippled retroviruses ought expose subtle requirements for ESCRT function.

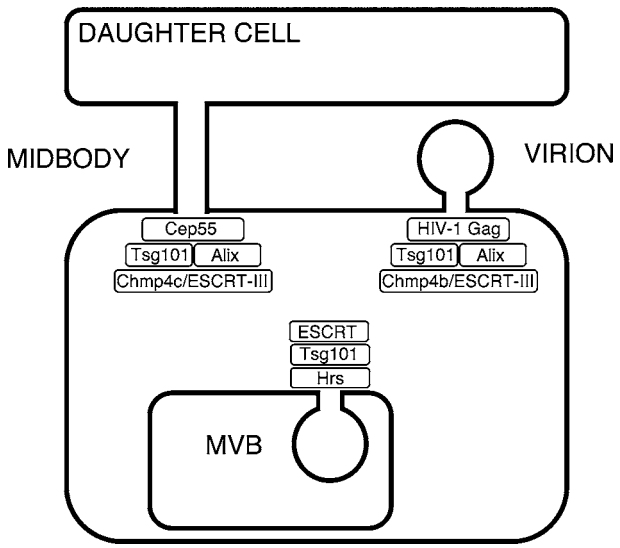
A recent report has also exposed an alternative L-domain-independent mechanism that HIV-1 can use to recruit Alix and make good its exit (Figure 1) [34]. This involves interaction of Alix's BroI domain with HIV-1 NC [34]. As well as requiring multimerization through Alix's C-terminus, the ability of Alix to rescue HIV-1 release depends upon the interaction of Alix's BroI domain with zinc fingers within HIV-1 NC. The introduction of point mutations into HIV-1 NC produces a defect in HIV-1 release that is similar to, albeit milder than, that observed upon disruption of HIV-1's PTAP L-domain [34]. These defects were not additive, suggesting that the same basic mechanism was compromised in each case. However, only the PTAP mutation within HIV-1 p6, not the zinc-finger mutation within NC, could be rescued through Alix overexpression, suggesting that the NC–BroI interaction may regulate the interaction of Alix with HIV-1's auxiliary L-domain. Interestingly, NC binds viral nucleic acids and, whereas the interaction of Alix with HIV-1 NC was independent of nucleic acid, the mutations in HIV-1 NC that compromise viral release severely disrupt packaging of genomic RNA into nascent virions [35], raising the possibility that defects in genome packaging may precipitate defects in viral release.

A new role for ESCRTs

The topological equivalence of MVB biogenesis and virion release allowed conceptual similarities to be explored, and recent data suggest that another cellular event bears a similar topological equivalence [23,36,37]. In the terminal stages of cell division, daughter cells must separate through the process of cytokinesis (Figure 2). Here, cells remain connected by a thin membranous stalk, the midbody, after mitosis; resolution of this midbody is required for completion of cell division. Recent studies have shown that the ESCRT machinery provides an activity allowing abscission to occur; here, the midbody resident, Cep55 (centrosome protein 55), recruits the ESCRT machinery through interaction with Tsg101 and Alix [23,36,37]. Cep55's hinge region binds to diproline motifs within Alix and Tsg101's proline-rich region [36,37], although whether these interactions are exclusive remains to be established. In both cases, however, disruption of Cep55–Tsg101 or Cep55–Alix interactions precipitates defects in cytokinesis, clearly demonstrating an essential role for these proteins in abscission [23,36,37]. Alix depletion has the greatest effect on cytokinesis [36,37] and Alix can bind numerous cellular partners [38]. Mutagenesis studies have demonstrated that only interaction of Alix with Cep55, Tsg101 and Chmp (charged multivesicular body protein) 4 are required for cytokinesis [23,37], suggesting that, via Cep55, Alix functions to recruit ESCRT-III to the midbody to achieve abscission. A secondary phenotype is observed in Alix- or Cep55-depleted

Figure 2 | Topological equivalences of ESCRT activity

Cartoon depicting sites of ESCRT activity and their topological equivalence. Hrs, hepatocyte growth factor-regulated tyrosine kinase substrate.



cells whereby midbody morphology is disrupted [23]. Microtubules persist through the central region of the midbody, but tubulin epitopes here are usually obscured by a proteinaceous density, resulting in a gap in anti-tubulin staining [39]. In Cep55- or Alix-depleted cells, or in cells reliant upon versions of Alix that either cannot be recruited to the midbody or cannot recruit ESCRT-III to the midbody, these central-region microtubules become exposed, suggesting that the ESCRT machinery, specifically ESCRT-III, may be a significant component of this density [23]. Thus we conclude that, just as endosomal Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) acts as an adaptor to recruit the ESCRT machinery to sites of MVB biogenesis and Gag proteins act as retroviral adaptors to recruit the ESCRT machinery to sites of viral assembly, Cep55 acts as a mitotic adaptor, recruiting the ESCRT machinery to the midbody to achieve abscission.

Conservation or acquisition

These data point to a fundamental cellular role for the ESCRT machinery; what is not presently clear is the extent to which this is a conserved or an acquired role. The mammalian ESCRT machinery is significantly expanded when compared with that of lower organisms such as *Saccharomyces cerevisiae* (Table 1). Given that there appears to be little current evidence to support a role for the ESCRT machinery in yeast cell division [yeast null for ESCRT components that function in metazoan cytokinesis display no overt growth phenotypes (M. Agromayor, J.G. Carlton, J.P. Phelan, K. Bowers, K. and J. Martin-Serrano, unpublished work)], one interpretation of this gene expansion is that it reflects the adaptation to different cellular functions. For example, whereas yeast contain a single copy of Snf7, mammalian cells express three orthologues: Chmp4a, Chmp4b and Chmp4c. Dominant-negative versions of ESCRT-III proteins inhibit cell division [23,36,37,40], yet, in the case of Chmp4s, YFP (yellow fluorescent protein)–Chmp4c is the most potent inhibitor of cytokinesis. Conversely, YFP–Chmp4b appears more potent than YFP–Chmp4a or YFP–Chmp4c at disrupting HIV-1 release [23]. These data suggest that expansion within the ESCRT machinery may have allowed for adaptation to mammalian-specific functionalities, such as cell division. However, although these data support the hypothesis that the ESCRT machinery plays an acquired role in cytokinesis, evidence from organisms such as *Arabidopsis thaliana*, where ESCRT-null plants exhibit cell division defects [41], argues that ESCRT function during cytokinesis is more of a conserved function. Recent reports have identified crenarchaeal ESCRT-III subunits and Vps4 homologues [42,43]. Archaea possess no endomembranes, and thus a comparative role in MVB sorting cannot easily be ascribed to these proteins. Indeed, an intriguing possibility is that archaeal ESCRT-III components function in bacterial cell division. Indeed, it could well be that the ESCRT machinery plays a more ubiquitous role in cytokinesis, with mammalian gene duplication representing a specialization of this process rather than an adaptation.

As with the role of the ESCRT machinery in viral release or MVB formation, the exact roles these proteins play in abscission remain to be established. Clearly this machinery must act from a cytosolic environment and the topological similarities of these three processes (Figure 2) leads to the suggestion that ESCRT proteins play a direct role in fusing the membranous stalks from the inside. Overexpressed ESCRT-III subunits can form tubular stalks *in vivo* [44], and recent biophysical studies have demonstrated that recombinant ESCRT-III subunits can assemble into helical tubules with their membrane-interacting surfaces facing outwards and Vps4-binding surfaces facing inwards [45], suggesting that they could assemble within the neck of an inwardly budding vesicle or viral stalk, or indeed within the lumen of the midbody, to provide an activity regulated by Vps4. Alternatively, the ESCRT machinery may co-ordinate downstream proteins to achieve membrane fusion, perhaps by localizing a fusion machinery or, in the case of the midbody, disassembling the microtubules. Whatever the outcome, the involvement of the ESCRT machinery in a new cellular process and the recent findings that crippled retroviruses can expose novel requirements for ESCRT function highlights a fruitful future for mechanistic studies of ESCRT action.

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