The emerging shape of the ESCRT machinery

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Abstract | The past two years have seen an explosion in the structural understanding of the endosomal sorting complex required for transport (ESCRT) machinery that facilitates the trafficking of ubiquitylated proteins from endosomes to lysosomes via multivesicular bodies (MVBs). A common organization of all ESCRTs is a rigid core attached to flexibly connected modules that recognize other components of the MVB pathway. Several previously unsuspected key links between multiple ESCRT subunits, phospholipids and ubiquitin have now been elucidated, which, together with the detailed morphological analyses of ESCRT-depletion phenotypes, provide new insights into the mechanism of MVB biogenesis.

Intralumenal vesicle
A small <50-nm vesicle that forms through inward budding of the endosomal membrane away from the cytosol.

Multivesicular body (MVB)
A morphologically defined endocytic organelle that is characterized by multiple internal vesicles in which internalized receptors are packaged before degradation.

Lysosome
Membrane-bound organelle in higher eukaryotic cells that has an acidic interior and is the major storage site of the degradative enzymes (acidic hydrolases) that are responsible for the breakdown of internalized proteins and many membrane proteins. Functionally equivalent to the yeast vacuole.

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Many plasma-membrane proteins are in a constant flux throughout the internal trafficking pathways of the cell. Some receptors, such as that for transferrin, are continuously internalized into recycling endosomes and returned to the cell surface. Others, such as the epidermal growth factor (EGF) receptor (EGFR), are sorted into intralumenal vesicles (ILVs) of morphologically distinctive endosomes that are known as multivesicular bodies (MVBs). These MVBs fuse with lysosomes, resulting in degradation of their cargo by lysosomal acidic hydrolases[1]. In yeast, an analogous pathway is involved in trafficking to the vacuole.

Endosomes can be operationally defined as being either early or late, referring to the relative time it takes for endocytosed material to reach either stage. However, both the structure and composition of endosomal compartments changes during the transition from early to late endosomes. Ultrastructural studies indicate that early endosomes are predominantly tubulovesicular structures, which constitute a major sorting platform in the cell, whereas late endosomes show the characteristics of typical MVBs and are capable of fusing with lysosomes[1]. The transition between these two stages is thought to occur by progressive involution of the limiting membrane to form ILVs and concomitant removal of recycling material via the fission of tubular elements that give rise to the recycling endosomes. Both the lipid and protein composition of the endosome changes along the pathway to lysosomes (BOX 1).

The best characterized signal for entering membrane proteins into the degradative MVB pathway is the ubiquitylation of these cargoes. At the centre of a vast protein–protein and protein–lipid interaction network that underpins ubiquitin-mediated sorting to the lysosome are the endosomal sorting complexes required for transport (ESCRTs) (FIG. 1), which are conserved throughout all major eukaryotic taxa (BOX 2).

Four distinct ESCRTs, known as ESCRT-0, -I, -II and -III, are recruited to endosomes through both protein and lipid interactions. ESCRT-0, -II and -III preferentially interact with 3-phosphoinositides that are enriched in endosomes, using different types of lipid-recognition domains (BOX 3). Although cell-fractionation studies suggest that the ESCRTs are predominantly cytosolic and become recruited to endosomes, quantitative ultrastructural studies of T cells and macrophages indicate that, at least in these cells, the majority (80%) of ESCRT-0 and ESCRT-I are membrane associated, with the greatest abundance (45%) on tubulovesicular structures[2].

ESCRT-0, -I and -II, which are enlisted early in the degradative MVB pathway, have ubiquitin-interacting modules that are necessary for cargo sorting (BOX 3). Curiously, although the ubiquitin-epitope that is recognized in each case is similar, no two ESCRT complexes use the same type of module to do so. By contrast, ESCRT-III, the final complex in the pathway, has no ubiquitin-recognizing module, but instead actively recruits de-ubiquitylating enzymes (DUBs) to remove ubiquitin from the cargo before incorporation into ILVs, and recruits the machinery to facilitate the disassembly of the ESCRTs from the endosomal membrane.

Recent structural studies of ESCRTs by X-ray crystallography and NMR have yielded detailed atomic models for the cores of ESCRT-I, -II and -III. All of the ESCRTs have a stable, multisubunit core to which domains that are crucial for their function in the MVB pathway are

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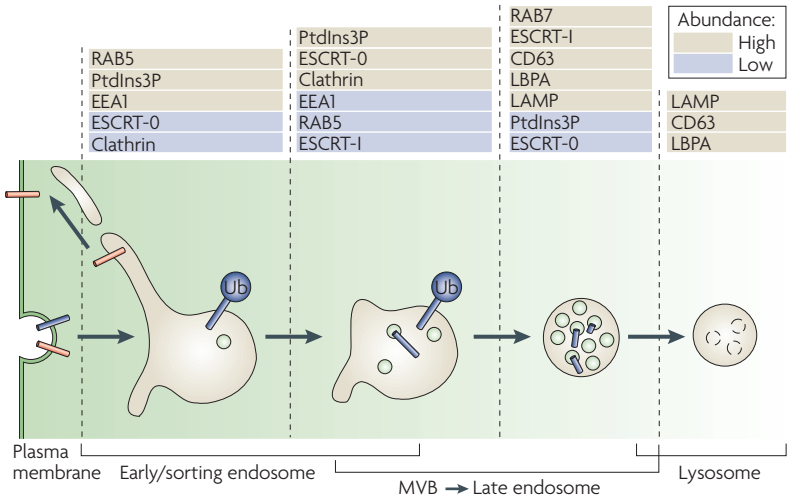
Box 1 | **An overview of the endocytic pathway**



Membrane proteins such as receptors with their bound ligands are removed from the plasma membrane by incorporation into endocytic vesicles that fuse with an early endosomal compartment (also known as a sorting endosome vesicles). Both cargoes that are destined for degradation and for recycling pass through the sorting endosome. Tubular elements from this tubulovesicular compartment undergo fission to become incorporated into recycling endosomes from which the cargo is returned to the plasma membrane. Ubiquitin (Ub) functions as the principal targeting signal to direct cargo incorporation into intralumenal vesicles (ILVs) to form multivesicular bodies (MVBs) that fuse with the late endosome. Early endosomes are characterized by high levels of phosphatidylinositol-3-phosphate (PtdIns3P) and the small GTPase RAB5, which cooperate to recruit the tethering molecule early endosome antigen-1 (EEA1)[18,124]. The progression from early to late endosomes is marked by replacement of RAB5 with RAB7 (REF. 124), and accumulation of a large number of 40–100-nm ILVs[23] enriched in PtdIns3P, cargo proteins (for example, epidermal growth-factor receptor (EGFR)) and tetraspannins (for example, CD63). In *Drosophila*, PtdIns(3,5)P_2 has been partially localized to an acidic endosomal compartment using a green fluorescent protein (GFP)-tagged lipid-binding protein[125]. Recent ultrastructural studies point to the existence of distinct subpopulations of MVBs; some of these contain the lipid lysobisphosphatidic acid (LBPA), the bulk of which is localized to lysosomes, and others are enriched in EGFR[126]. Although incorporation of cargo appears to be coordinated with ILV formation, studies in yeast suggest that these processes can be uncoupled[80]. LAMP, lysosome-associated membrane protein.

Ubiquitylation
Covalent attachment of ubiquitin via its C-terminal Gly to Lys side chains in target proteins. Ubiquitin can form seven distinct polyubiquitin chains through seven internal Lys residues.

Endosomal sorting complex required for transport
(ESCRT). A multimeric protein complex that was first identified biochemically in yeast. ESCRTs control the sorting of endosomal cargo proteins into internal vesicles of multivesicular bodies.

ESCRT-0
Nomenclature for the HRS–STAM complex, which was proposed as a recent extension of the originally defined ESCRT-I, -II and -III.

flexibly connected. ESCRT-I and -II form complexes with a well defined stoichiometry, which is evident in the structures of the cores. Although the structure of only one subunit has been determined for ESCRT-III, other ESCRT-III subunits are likely to have closely related folds. Progress in atomic-resolution structures has been paralleled by ultrastructural studies of cells depleted of ESCRT components (summarized in TABLE 1).

Many of the ESCRT-associated proteins have alternative cellular roles beyond lysosomal trafficking. For example, elements of the ESCRT machinery are also recruited by retroviruses such as HIV-1, murine leukaemia virus (MLV) and equine infectious anaemia virus (EIAV) for budding from the plasma membrane, a process that is topologically identical to budding away from the cytosol to form MVBs[3]. These alternative roles have been reviewed elsewhere[4–6], and here we will focus on the structures of the four ESCRT complexes from both yeast and mammalian cells and their functions in the trafficking of ubiquitylated cargoes and the formation of ILVs in the MVB pathway.

ESCRT-0

HRS (hepatocyte growth factor (HGF)-regulated Tyr-kinase substrate; Vps27 in yeast) and STAM (signal transducing adaptor molecule; Hse1 in yeast) make up ESCRT-0, which is essential for initial selection of ubiquitylated cargo at the endosomal membrane. ESCRT-0 associates with endosomes through the interaction of the HRS FYVE (Fab1, YOTB, Vac1 and early endosome antigen-1 (EEA1)) domain with phosphatidylinositol-3-phosphate (PtdIns3P), where it in turn recruits clathrin and sequesters ubiquitylated cargo in clathrin-coated microdomains[7]. HRS also has an important role in recruiting the downstream ESCRTs by direct interaction with the ESCRT-I component TSG101 (REF. 8).

Morphology of ESCRT-0 mutants. Deletion of yeast ESCRT-0 subunits gives rise to a class E phenotype[9], which is characterized by the missorting of both biosynthetic and endocytic cargo to an aberrant prevacuolar, multilamellar compartment[10,11]. *Drosophila melanogaster* larvae that express a mutant form of HRS also have defects in endosomal membrane invagination and ILV formation[12]. HRS-deficient mice or a double knockout of the two major isoforms of STAM, STAM1 and STAM2, are embryonic lethal (E11 and E10.5, respectively) and yield fibroblasts with aberrantly enlarged vacuolar structures[13,14]. Ultrastructural analysis of mammalian cells that are depleted of HRS by small interfering (si)RNA show that these vacuoles contain few internal vesicles[15]. However, growth-factor-receptor degradation might not be completely contingent on internal vesicle formation[15,16], as the degradation of activated EGFR[17] or internalized EGF[15] is only partially impaired in HRS-depleted cells, and degradation of MET, the receptor for HGF, is only modestly delayed[16].

Recruitment of ESCRT-0 to the endosomal membrane. The N-terminal FYVE domain of HRS mediates recognition of PtdIns3P, a highly abundant phosphoinositide in endosomal membranes[18–20]. This FYVE domain is the most specific of the three phosphoinositide-recognition modules that are used in the ESCRT pathway.

HRS also binds ubiquitin via a ubiquitin-interacting motif (UIM), which is essential for efficient sorting of ubiquitylated membrane proteins[21,22] (BOX 3). The overlapping recognition surface on ubiquitin for all of the ESCRTs means that a given ubiquitin moiety can interact with only one ESCRT at a given time. There is no pattern in the affinities of the interactions to impart an intrinsic directionality in cargo handover (that is, from ESCRT-0 to -I to -II). Although the initial data from yeast suggest a sequential arrangement of these complexes, one may also envisage that cargoes are stationary clusters to which multiple ESCRTs are recruited in a stochastic manner, thereby collectively enabling their retention and concentration in microdomains.

HRS and the endosomal clathrin coat. Ubiquitylated cargo and ESCRT components are not uniformly distributed on endosomal surfaces. Clathrin acts as an organizer of the ESCRT pathway. HRS is highly enriched (~20-fold)

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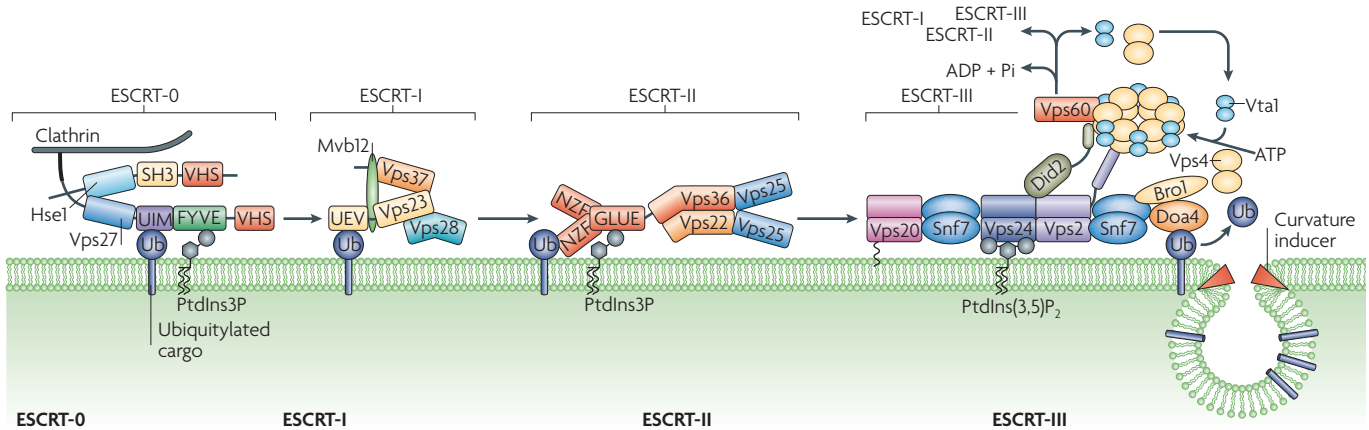

ESCRT-0

S. cerevisiae	Mammalian
Vps27	HRS (HGS)
Hse1	STAM1, STAM2

ESCRT-I

S. cerevisiae	Mammalian
Vps23	VPS23 (TSG101)
Vps28	VPS28
Vps37	VPS37A, B, C, D
Mvb12	?

ATPase complex

S. cerevisiae	Mammalian
Vps4	VPS4A and VPS4B (SKD1)
Vta1	LIP5

ESCRT-II

S. cerevisiae	Mammalian
Vps22	VPS22 (EAP30, SNF8)
Vps25	VPS25 (EAP20)
Vps36	VPS36 (EAP45)

Accessory

S. cerevisiae	Mammalian
Bro1	ALIX (AIP1, PDCD6IP)
Doa4	UBPY?
?	AMSH

ESCRT-III

S. cerevisiae	Mammalian
Vps2	VPS2A, B (CHMP2A, B)
Vps24	VPS24 (CHMP3)
Snf7	SNF7-1, -2, -3 (CHMP4A, B, C)
Vps20	VPS20 (CHMP6)
Did2	CHMP1A, B
Vps60	CHMP5
-	CHMP7

Figure 1 | **A schematic of the ESCRT machinery involved in sorting cargo to lysosomes via MVBs.** The four ESCRTs are recruited to endosomes by their interactions with membranes, clathrin, ubiquitin (Ub) and with each other. Features of both yeast and mammalian pathways are included. Lipid recognition of either phosphatidylinositol-3-phosphate (PtdIns3P) by the FYVE domain of Vps27 (ESCRT-0) or the GLUE domain of Vps36 (ESCRT-II), or PtdIns(3,5)P_2 by Vps24 (ESCRT-III) might contribute to the early or late endosomal localization of the components (BOX 3). All of the ESCRTs except ESCRT-III recognize and bind the ubiquitylated cargo, either through a ubiquitin-interacting motif (UIM) (ESCRT-0), a ubiquitin E2 variant (UEV) domain (ESCRT-I) or the GLUE domain of Vps36 (ESCRT-II). ESCRT-III orchestrates the last steps in the pathway in which ubiquitin is removed by a de-ubiquitinase (degradation of alpha-4 (Doa4)), and the complexes are disassembled by the AAA+ ATPase Vps4. Budding away from the cytosol is depicted as being facilitated by a curvature-inducing factor that could flex the membrane by being localized to the neck of the budding vesicle. The ESCRT components might facilitate the recruitment of such curvature-inducing factors or the concentration of inverted cone-shaped components in the endosomal membrane, such as lysobisphosphatidic acid[135] (LBPA) (with the caveat that yeast does not produce LBPA), and non-cargo transmembrane proteins that have bulky glycosylations on the lumenal side of the membrane, such as tetraspannins. Did2, Vps2 and Vps60 are shown interacting with the Vps4–Vta1 complex (FIG. 4). The bottom panels list ESCRT subunits and accessory proteins from *Saccharomyces cerevisiae* and their mammalian homologues. AMSH, associated molecule with the SH3 domain of STAM; CHMP, charged multivesicular body (MVB) proteins; SH3, Src-homology-3; STAM, signal transducing adaptor molecule; UBPY, ubiquitin-specific protease Y.

De-ubiquitylating enzyme
Protease that can cleave the isopeptide bond between ubiquitin and Lys side chains of ubiquitylated substrates.

Early endosome antigen-1
A protein that plays a part as a tether in the homotypic fusion of early endosomes.

Phosphatidylinositol-3-phosphate
(PtdIns3P). A phospholipid that is highly enriched in endosomal membranes. Several ESCRT proteins encode PtdIns3P-binding domains, which allow these proteins to be recruited to endosomes.

Clathrin
Coat protein that decorates clathrin-coated vesicles and is recruited by ESCRT-0 to sorting endosomes where it forms a flat double-layered coat, which is thought to sequester ubiquitylated cargo prior to incorporation into intralumenal vesicles.

in clathrin-coated 80–500-nm diameter microdomains that decorate the endosomal membrane[21,23,24], although at least in macrophages and T cells HRS is also detected on tubulovesicular membranes that are closely apposed to both early and late endosomes[2]. Endosomes that harbour an HRS–clathrin coat are characterized by a low number of internal vesicles (less than 5) and are therefore variably referred to as early endosomes or MVBs[23,24]. HRS binds clathrin by a Leu-Ile-Ser-Phe-Asp motif at its C terminus. The other component of ESCRT-0, STAM, also binds directly to clathrin; however, the binding site has not been mapped[25]. HRS recruits clathrin to endosomes whereas, conversely, clathrin binding restricts HRS to microdomains that are dynamically exchanging with the cytosol and are required for efficient EGF degradation[26].

The flat clathrin coat that is recruited by HRS has a double-layered appearance[24]. Immuno-electron microscopy (EM) indicates that HRS is located at or near the membrane in a thin electron-dense layer, whereas clathrin localizes more peripherally in a fuzzy, less dense layer[24]. The molecular composition of these HRS–clathrin coats is still under investigation, but it is thought to be the first point of engagement of ubiquitylated cargo with the ESCRT machinery[7,24,26]. The clathrin scaffold that restricts HRS distribution might also concentrate ubiquitylated cargo into microdomains where the cargo can more readily encounter ESCRT-I.

The structure of HRS. STAM and HRS interact constitutively with each other through coiled-coil regions[27,28], and expression of STAM is strictly contingent on the presence of HRS[14,29,30]. The atomic-resolution structures of only the VHS (Vps27, HRS and STAM) and FYVE domains of HRS have been determined[31] (FIG. 2). Both ESCRT-0 components have VHS domains. These domains are common N-terminal elements in proteins that are involved in intracellular trafficking and might play a part in cargo binding. The VHS domain of HRS has been proposed to participate in an alternative

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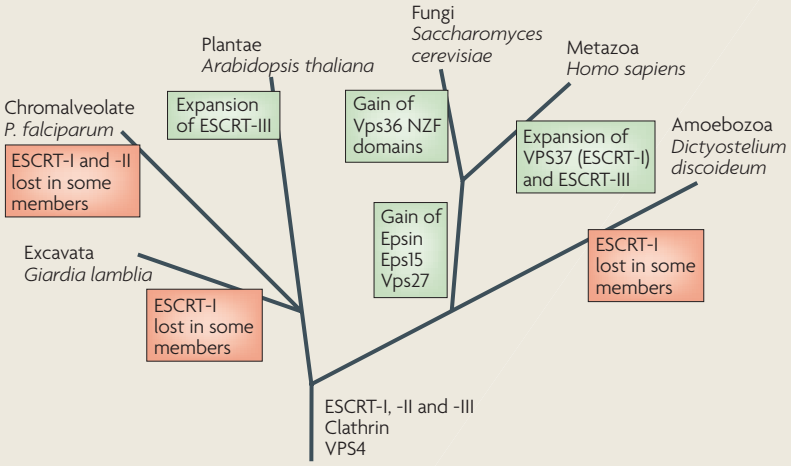
Box 2 | **Evolution of the ESCRTs**



The components of the multivesicular body (MVB) pathway are ancient and predate the divergence of plants and animals more than one billion years ago[127,128]. ESCRT-I, -II and -III are present in all major eukaryotic taxa (a single species with a sequenced genome from each clade is shown in italics). However, some members of the clades have subsequently lost specific ESCRT components. For example, ESCRT-I and ESCRT-II subunits are absent from *Plasmodium falciparum* and the related parasite *Toxoplasma gondii*, but these organisms have Vps4 and ESCRT-III homologues and appear to form MVBs[129]. There is no sequenced eukaryotic genome without an ESCRT-III-related gene[127]. This indicates that the minimal ESCRT machinery necessary for formation of intralumenal vesicles (ILVs) might be ESCRT-III.

ESCRT-0 seems to be a relatively recent addition that has arisen in the last common ancestor of the fungi and metazoa. Nevertheless, many of the endomembrane components with which ESCRT-0 interacts, such as clathrin, EPS15 and ESCRT-I, are more ancient[127]. This indicates that ESCRT-I is recruited by other means in other taxa. A more recent development in the ESCRT components are the Npl4-type zinc-fingers (NZFs) in the ESCRT-II VPS36 GLUE domain, which recognize ubiquitylated cargo and ESCRT-I. If the ESCRT pathway is viewed as a sequence from ESCRT-0 through ESCRT-III, there appears to be no example characterized in which an upstream component is present but downstream components are absent[127,128].

The size of the subset of ESCRT-III-related genes is greatly expanded in mammals relative to yeast. Similarly, there are four mammalian isotypes of the ESCRT-I subunit VPS37 and this subunit is highly divergent among species. By contrast, most eukaryotes have a single paralogue of the ESCRT-II subunit VPS25 (REF. 128).

ESCRT-independent role of HRS in the rapid recycling of membrane receptors to the plasma membrane, although relevant binding partners remain elusive[32]. Interestingly, the VHS domain of STAM has been reported to interact with ubiquitin and might therefore contribute, together with the UIM of STAM, to cargo selection[9,33].

The HRS FYVE is a double zinc-finger domain[31,34] (FIG. 2). Although the HRS FYVE domain structures have no bound phosphoinositide, the inositide-containing structure of the FYVE domain from EEA1 provides a good model for lipid binding, showing that phosphoinositide recognition involves an Arg-(Lys/Arg)-His-His-Cys-Arg motif in strand β1 (REF. 35), and suggesting that hydrophobic residues that are exposed on the surface of the domain enable partial penetration into the membrane[36]. These domains typically bind lipids with low affinity and can only target PtdIns3P-enriched membranes in cells when they are part of oligomeric complexes.

A recent 16-Å resolution cryo-EM structure of recombinant HRS indicates that it forms a hexameric cylinder[37]. Given the absence of STAM in this structure, it is not clear how the hexamer relates to the biological assembly.

Interaction of ESCRT-0 with ESCRT-I. HRS and its yeast orthologue, Vps27, bind directly to ESCRT-I, using their Pro-(Ser/Thr)-X-Pro (in which X represents any amino acid) motifs and the ubiquitin E2 variant (UEV) domain of the tumour susceptibility gene-101 (TSG101; Vps23 in yeast) subunit of ESCRT-I[38–44]. Although these motifs interact with ESCRT-I, the affinity is relatively weak (about 140 μM) and additional uncharacterized interactions probably stabilize the complex[42]. Vps27 and Vps23 association is restricted to membranes[43], and this might confine activation of ESCRT-I to endosomal membranes.

ESCRT-I

Saccharomyces cerevisiae ESCRT-I was originally characterized as a heterotrimeric complex of Vps23, Vps28 and Vps37 (FIG. 3a)[45]. More recently, several groups have characterized Mvb12 (REFS 46–49), an additional ESCRT-I subunit that interacts with the heterotrimeric complex with a 30-nM affinity[49]. Human ESCRT-I consists of TSG101, VPS28 and one of four isotypes of VPS37 (VPS37A–D)[50–53]. The human orthologue of Mvb12 is currently unknown.

Morphology of ESCRT-I mutants. In yeast, deletion of any ESCRT-I subunit gives rise to a class E phenotype, except for deletion of *MVB12,* which causes only a mild phenotype with a partial sorting defect[46–48]. In mammalian cells, depletion of TSG101 has a much stronger inhibitory effect on receptor degradation than HRS depletion and causes a strikingly different endosomal morphology[15,54]; whereas depletion of HRS leads to enlarged vacuolar structures with EGFR trapped at the limiting membrane, depletion of TSG101 promotes the accumulation of EGFR and EGF on extensive tubular clusters and induces the formation of multicisternal structures with an internal matrix[15,54]. This phenotype may suggest that, in contrast to HRS, TSG101 (ESCRT-I) is required for the maintenance of the vacuolar morphology of endosomes[15]. The tubules colocalized with the early endosomal markers transferrin and EEA1, but not with lysosome-associated membrane protein-1 (LAMP1), a marker for late endosomes and lysosomes[15]. Although the extensively tubulated phenotype is reminiscent of the morphology of recycling endosomes, depletion of TSG101 did not affect the distribution of RAB11, suggesting that these tubules do not simply reflect an expansion of the perinuclear recycling compartment[15].

The structure of the ESCRT-I core. ESCRT-I is structurally organized in a core complex with flexibly connected modules that mediate interactions with other partners such as ESCRT-0, ubiquitin, the ESCRT-associated protein ALIX (also called AIP1) and, in yeast, with ESCRT-II. The ESCRT-I core is built from three helical hairpins

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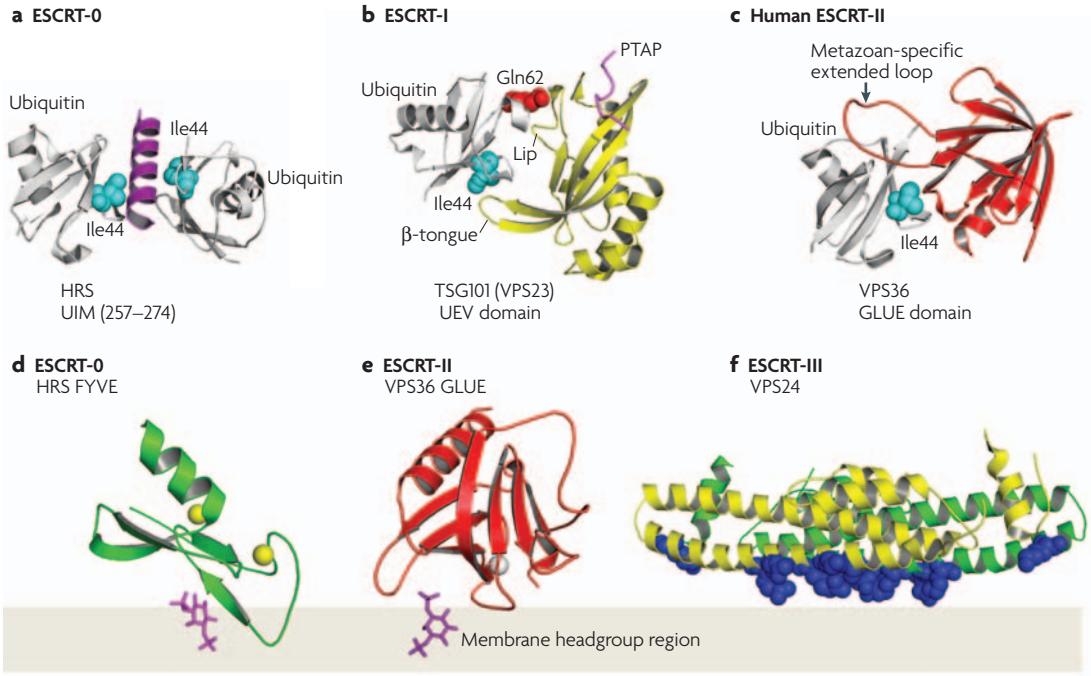
Box 3 | **Ubiquitin and phosphoinositide recognition in the ESCRT pathway**



	ESCRT-0	ESCRT-II	ESCRT-III
Lipid preference	PtdIns3P	PtdIns3P	PtdIns(3,5)P_2
Binding specificity	High	Intermediate	Low

The affinity of all ubiquitin-binding domains (UBDs) of the multivesicular body (MVB) pathway, including those of ubiquitin-interacting motifs (UIMs), are weak, ranging from 100–500 μM[59,67,70,130]. These weak associations might form high-avidity interactions with polyubiquitylated or multiply-monoubiquitylated proteins[131]. All of the ESCRT UBDs interact with the same surface of ubiquitin centred on Ile44 (cyan spheres), suggesting either a sequential pathway for cargo handover or a mechanism to concentrate cargo in regions that contain multiple types of ESCRTs.

The HRS (hepatocyte growth factor regulated Tyr-kinase substrate) UIM is a single α-helix that can interact with two ubiquitins that are arranged on opposite sides of the helix (**a**; Protein Data Bank (PDB) ID 2D3G[132]). Both ubiquitin-binding sites are necessary for efficient degradation of internalized receptors. By contrast, yeast Vps27 has two tandem UIMs that each bind only one ubiquitin, with the N-terminal UIM having a higher affinity[133]. ESCRT-I recognizes ubiquitin with a ubiquitin E2 variant (UEV) domain that has a fold similar to a E2 ubiquitin-conjugating enzyme (**b**; PDB ID 1S1Q[59]). The HIV-1 PTAP peptide (purple) bound to the UEV domain (PDB ID 1M4P[40]) represents the binding of the HRS (Vps27 in yeast) Pro-(Ser/Thr)-X-Pro motif (in which X represents any amino acid) from ESCRT-0 to ESCRT-I. The UEV also recognizes a second hydrophilic patch that is centred on Gln62 (red spheres) on ubiquitin in addition to the Ile44 patch[44,58]. Yeast ESCRT-II recognizes ubiquitin with an Npl4-type zinc finger (NZF) domain. Remarkably, metazoan ESCRT-II GLUE domains lack the NZF insertion, but bind ubiquitin directly by the GLUE domain along the edge of the β-sandwich (**c**; PDB ID 2HTH[69]).

There are three phosphoinositide-recognition domains in the ESCRT pathway: the FYVE domain of ESCRT-0 (**d**; PDB ID 1VFY[134]; yellow spheres represent bound zinc atoms), the GLUE domain of ESCRT-II (**e**; PDB ID 2CAY[56]; white sphere represents location of fungal-specific NZF insertion) and the VPS24 (CHMP3) subunit of ESCRT-III (**f**; PDB ID 2GD5 (REF. 82)). Basic residues interacting with membranes are shown as blue spheres. The HRS FYVE domain with the narrowest specificity (phosphatidylinositol-3-phosphate (PtdIns3P) binding) recruits ESCRT-0 to early endosomes. The more broadly specific VPS24 and GLUE domain of ESCRT-II localize to later endosomes. PtdIns3P in the binding sites for the FYVE and GLUE domains is modelled. PtdIns(3,5)P_2, phosphatidylinositol-3,5-bisphosphate.

that are arranged like a fan with Vps23 in the centre and Vps28 and Vps37 on either side (FIG. 3a)[55,56]. The central role of the Vps23 hairpin in mediating the intersubunit interactions is consistent with the crucial role of Vps23 in maintaining the stable expression of Vps28 and Vps37 (REF. 57). Equilibrium centrifugation of the recombinant, full-length, four-subunit yeast ESCRT-I shows a 104-kDa complex with a 1:1:1:1 stoichiometry[49]. In yeast cytosol, ESCRT-I appears to form higher-order oligomers that are mediated by the Mvb12 subunit[47].

The interaction of ESCRT-I with ubiquitin. ESCRT-I binds ubiquitin through the N-terminal UEV domain of Vps23 (TSG101). There are two adjacent regions of the UEV domain that interact with a bound ubiquitin[58,59] (BOX 3), and these regions cooperate to increase the efficiency of ubiquitylated cargo sorting[44]. This broader area of interaction could be the consequence of Vps23 (TSG101) having evolved a larger ubiquitin-interacting domain to accommodate both ubiquitin and Vps27 (HRS) recognition.

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Table 1 | **Effect of depletion of the metazoan MVB-pathway components**

Component	Effect of depletion on endosome morphology	Step affected by depletion or knockout	Effect of depletion on sorting
VPS34	Large vacuoles with few internal vesicles[136,137]	Internal vesicle formation	Does not prevent sorting to lysosomes[137]. In yeast, results in decreased carboxypeptidase Y activity[138]
HRS (ESCRT-0)	Enlarged vacuoles with few internal vesicles[14,15]	Internal vesicle formation	Mildly impaired EGFR and MET downregulation[12,15,16,132]
STAM (ESCRT-0)	Swollen endosomes[14]	Not characterized	Impaired EGFR degradation[14]
TSG101 (ESCRT-I)	Extensive tubular pericentriolar cluster and multilamellar cisternae with intercisternal matrix[15,54]	Stabilization of vacuoles and internal vesicle formation	EGFR accumulation in tubular clusters, prevents EGFR degradation[15,54]. Prolonged EGF signalling[98]
VPS37A (ESCRT-I)	Not characterized	Not characterized	Strongly delays EGFR degradation[50]
VPS36 (ESCRT-II)	Not characterized	Not characterized	Impaired degradation and lysosomal localization of EGFR[61]. No effect on EGFR[75]. No effect on MHC-I degradation[61]
VPS24 (ESCRT-III)	EGFR-containing MVBs with smaller diameter[98]	MVB fusion with lysosomes/late endosomes[98]?	Impairs EGFR degradation. Does not affect silencing of EGFR signalling[98]
CHMP5	Enlarged endosomes filled with internal vesicles[99]. Enlarged EGFR-containing vesicles[96]	MVB fusion with lysosomes?	Impaired degradation of endocytosed fluid-phase marker. Impaired degradation of TGFβ receptor. Hyperactivated TGFβ stimulation. Accumulation of TGFβ receptor at the plasma membrane[99]. Reduced rate of EGFR degradation and increase in the release of infectious HIV-1 (REF. 96)
LIP5 (Vta1 in yeast) [96]	LIP5 depletion has no effect on morphology[96]	VPS4 assembly/catalysis	Reduced rate of EGFR degradation and reduced release of HIV-1 (REF. 96)
AMSH	No dramatic morphological change	Sorting into ILVs[84]	Enhanced rate of EGFR degradation[75,85]
UBPY	Increased MVB number and volume. MVBs stitched together with regularly spaced repeating unit[120]	Stability of STAM and HRS, and MVB fusion with lysosome/late endosomes	Increase in ubiquitylated proteins, but no effect on levels of free ubiquitin. Inhibition of EGFR and MET degradation. Deubiquitylation of EGFR severely impaired[75,120]

AMSH, associated molecule with the Src-homology-3 (SH3) domain of STAM; EGF, epidermal growth factor; EGFR, EGF receptor; ILV, intralumenal vesicle; HRS, hepatocyte growth factor regulated tyrosine Tyr-kinase substrate; MHC-I, major histocompatibility class-I; MVB, multivesicular body; STAM, signal transducing adaptor molecule; TGFβ, transforming growth factor-β; UBPY, ubiquitin-specific protease Y.

The interaction of ESCRT-I with downstream ESCRTs. In yeast, the link between ESCRT-I and ESCRT-II is formed by the Vps28 C-terminal domain (a four-helix bundle[49,60]) binding with nanomolar affinity to a zinc-finger domain of Vps36 (REFS 49,56) (see below). Deletion of the C terminus of Vps28 causes a strong class E phenotype in yeast[55], indicating an important role for this ESCRT-I–ESCRT-II link (FIG. 3a). By contrast, mammalian VPS36 lacks the zinc finger, and no ESCRT-I–ESCRT-II link has yet been observed in mammalian cells. However, yeast two-hybrid analysis detects both TSG101–VPS22 and TSG101–VPS36 interactions[61].

ESCRT-II

ESCRT-II consists of Vps22, Vps25 and Vps36 (REF. 62). The core of ESCRT-II is a flat y-shaped heterotetramer with one copy each of Vps22 and Vps36 forming the base of the y and two copies of Vps25 forming the arms (FIG. 3b)[63,64]. All of the subunits of ESCRT-II have two tandem repeats of a winged-helix domain[63–65], a common type of protein–protein and protein–DNA interaction module. The C-terminal winged-helix domain of Vps25 provides a direct link to ESCRT-III by interacting with Vps20 both in yeast and mammalian cells (REFS 61,63,66). This might be an important trigger for ESCRT-III assembly.

GLUEing membranes, ubiquitin and ESCRT-I. The N-terminal domain of Vps36, an extension outside the ESCRT-II core, has been termed a GLUE domain[67] (GRAM-like ubiquitin-binding in EAP45) and has a typical pleckstrin homology (PH) fold[56,68,69] (FIG. 3b). Vps36 has an ancient origin and was probably present in the common ancestor of plants and animals. However, fungal Vps36 has acquired an insertion that consists of tandem N- and C-terminal Npl4-type zinc-finger (NZF-N and -C, respectively) domains between strands β6 and β7 of the PH fold. The GLUE domain in yeast preferentially interacts with PtdIns3P[56], which binds in a non-canonical, basic pocket that is distinct from the phosphoinositide-binding pocket that has been characterized in most other PH domains. The structure of the mammalian GLUE domain is also consistent with a non-canonical pocket for lipid binding, analogous to yeast[68,69].

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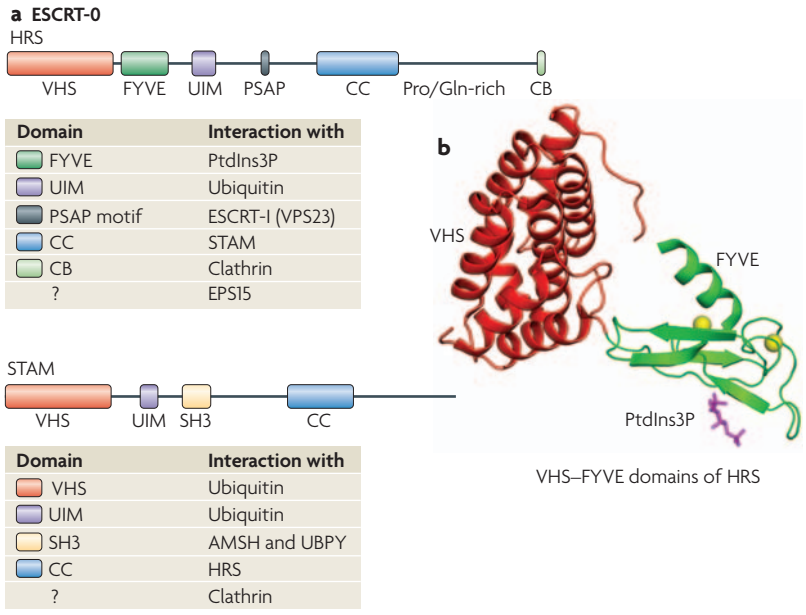

Figure 2 | **The organization of ESCRT-0. a** | Domain organization and interactions of the components of ESCRT-0, HRS (hepatocyte growth factor regulated Tyr-kinase substrate) and STAM (signal transducing adaptor molecule). **b** | The structure of the tandem VHS–FYVE domains of HRS (Protein Data Bank ID 1DVP[31]). The zinc-binding (yellow spheres) FYVE domain is important for recruiting the HRS–STAM complex to membranes through binding phosphatidylinositol-3-phosphate (PtdIns3P). AMSH, associated molecule with the SH3 domain of STAM; CB, clathrin-binding domain; CC, coiled-coil domain; SH3, Src-homolgy-3; UIM, ubiquitin-interacting motif; UBPY, ubiquitin-specific protease Y.

The yeast-specific insertion in the GLUE domain of Vps36 includes a zinc-finger domain (NZF-C) that binds ubiquitin and is important for efficient cargo sorting[70].The Vps36 NZF-C domain has a classic zinc-ribbon fold with the zinc atom held by four Cys residues contained in two loops. In yeast, mutations in the NZF-C that eliminate interaction with ubiquitin result in missorting of vacuolar hydrolases. Surprisingly, the human GLUE domain lacks this NZF insertion but still binds ubiquitin *in vitro*[67], and the structure of this complex shows ubiquitin bound to the PH domain itself[68,69]. Ubiquitin binding might therefore be a core function of this complex, even though the UBDs are not structurally conserved. In yeast, ubiquitin can bind simultaneously with PtdIns3P to the GLUE domain[56], and this is likely to be a feature that is conserved in metazoan GLUE domains[67–69].

Recombinant yeast ESCRT-II binds ESCRT-I with 30-nM affinity[49]. This is accomplished by the NZF-N in the Vps36 GLUE-domain insertion, which interacts with the Vps28 subunit from ESCRT-I (FIG. 3). This interaction is necessary for efficient sorting[49]. Curiously, in yeast cytosol, ESCRT-I and -II are distinct complexes[45,62], suggesting that their interaction is confined to endosomal membranes. Deletion of *MVB12* results in constitutive association of ESCRT-I and -II[47]. However, Mvb12 has no influence on the ESCRT-I–ESCRT-II association *in vitro*, indicating a more complex regulation of these interactions in cells[49].

The role of ESCRT-II in the MVB pathway. In yeast, ESCRT-II transiently associates with endosomes and facilitates recruitment of ESCRT-III components to membranes[62]. Deletion of any of the ESCRT-II components results in missorting of vacuolar hydrolases and their accumulation in class E compartments. Uncovering the role of ESCRT-II in metazoa has been more difficult. As in yeast, ESCRT-II in *D. melanogaster* has an important role in MVB trafficking of membrane receptors. Mutations in *D. melanogaster Vps25* cause an upregulation of the Notch signalling pathway[71–73]. This inappropriate activation of Notch signalling appears to be the consequence of accumulation of the membrane receptor Notch on aberrant endocytic structures where signalling from the receptor is not terminated. Sustained Notch signalling results in overexpression and secretion of the cytokine Upd, causing the proliferation of neighbouring cells. A similar phenotype is seen for mutants of the *D. melanogaster* homologue of Vps23, *erupted*[74]. *Vps23*, *Hrs* and *Vps25* mutants all result in accumulation of ubiquitylated proteins in aberrant endosomes.

In mammalian cells, however, siRNA-mediated depletion of ESCRT-II has no impact on the degradation of the ubiquitylated major histocompatibility class-I (MHC-I) complex[61,75], but strongly inhibits the downregulation of the cell-surface receptor ferroportin, which is regulated by the hormone hepcidin[61]. Both ESCRT-II-dependent[61] and independent[75] degradation of EGFR and EGF, respectively, have been reported. Collectively, these findings indicate that mammalian cells might have evolved cargo-specific MVB pathways, some of which might bypass ESCRT-II. Yeast two-hybrid interaction data indicate that ALIX might offer an alternative link between ESCRT-I and -III; however, depletion of ALIX, either on its own or in combination with ESCRT-II, does not affect MHC-I or EGF degradation[75].

ESCRT-III and related proteins

The ESCRT-III proteins in yeast consist of Vps2, Vps24, Snf7 and Vps20 (REF. 76), which are thought to form two subcomplexes. Two additional proteins that have sequences closely related to the ESCRT-III proteins, Did2 and Vps60, also have a role late in the MVB pathway[77–80]. The mammalian CHMP7 (charged MVB proteins or chromatin modifying protein-7), which has two tandem repeats of ESCRT-III-like sequences, has no fungal equivalent[81]. The functional significance of multiple isoforms of some mammalian ESCRT-III subunits has not been explored, and we will refer to all of these proteins collectively as ESCRT-III-related proteins.

All of the ESCRT-III-related proteins have a similar organization, with an N-terminal basic and a C-terminal acidic region. The recently determined crystal structure of the truncated human VPS24 (residues 9–183 from the 222-residue protein) reveals an organization that is likely to be common to all of the ESCRT-III-related proteins[82] (FIG. 4a). The core (residues 9–140) consists of an asymmetrical antiparallel four-helix bundle, with the first two helices forming a 70-Å hairpin. This hairpin forms two types of dimer interfaces (FIG. 4b), primarily using residues from helix α2. The connection to helix α5 from the C-terminal region is disordered.

Vacuolar hydrolases
Enzymes that are responsible for protein degradation in late endosomes and lysosomes.

Class E compartment
Aberrant multilamellar or multicisternal membrane-bound compartment in which both endocytic and biosynthetic proteins *en route* to the vacuole accumulate in Vps-mutant yeast of the class E subtype.

CHMP
A family of structurally related proteins with a basic N terminus and an acidic C terminus that interact with each other to form ESCRT-III. Some CHMPs have also been associated with nuclear functions.

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The C-terminal portion of VPS24 constitutes the autoinhibitory region (roughly from residue 150 to the C terminus), comprising helix α5 (which is present in the crystal structure) and the C-terminal microtubule-interacting and transport (MIT)-interacting region (named MIR), which is not present in the crystal structure. The autoinhibitory region inhibits hetero- or homodimerization by forming a competing interaction

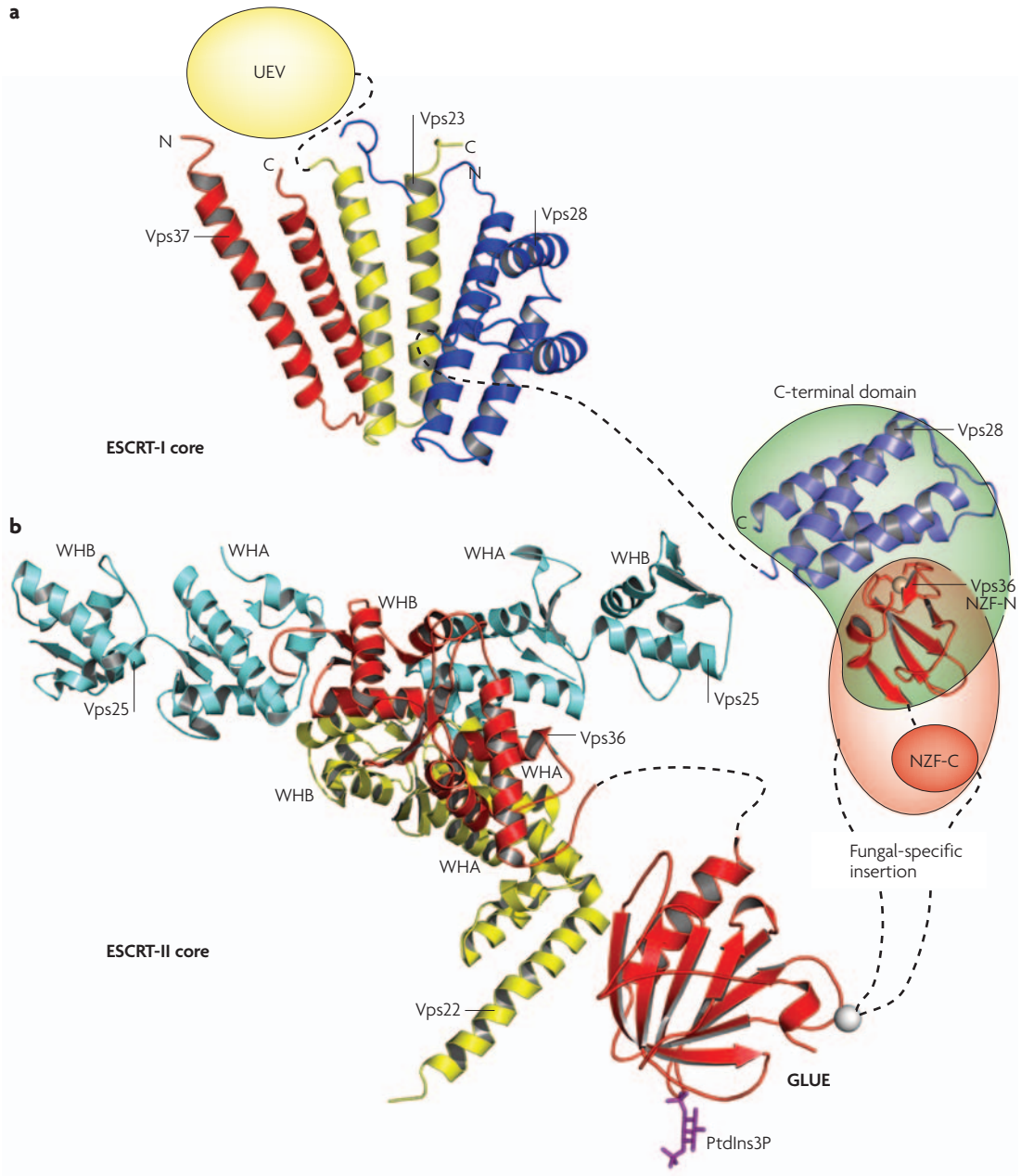

Figure 3 | **The architecture of the cores of ESCRT-I and ESCRT-II.** Both ESCRT-I and –II cores are built from repetitive structural units, despite having no internal sequence repeats. **a** | The yeast ESCRT-I core (Protein Data Bank (PDB) ID 2F66 (REF. 55) shown) is made of helical hairpins from three subunits: Vps23, Vps28 and Vps37. The tight core functions as a lynchpin to tether the ubiquitin-recognizing ubiquitin E2 variant (UEV) domain (yellow), to the ESCRT-II-recognizing C-terminal domain of Vps28 (green)[49]. **b** | The y-shaped rigid core complex of ESCRT-II (PDB ID 1W7P[63]) and its flexibly attached extensions tie together ubiquitin, ESCRT-I and ESCRT-III interactions. Each ESCRT-II subunit in the core is built from tandem repeats of winged-helix domains (WHA and WHB). The presence of two copies of Vps25 subunits, each of which can interact with the Vps20 subunit of ESCRT-III (BOX 2), indicates that ESCRT-II (recruited to membranes by its GLUE domain) might function as a branch point in the ESCRT pathway and nucleate networks of interactions on endosomal membranes (FIG. 4). Yeast has a fungal-specific insertion in the GLUE domain of Vps36 (red), composed of the N-terminal Npl4-type zinc finger (NZF-N) domain that recognizes Vps28 of the upstream ESCRT-I (PDB ID 2J9U[49]), and the C-terminal NZF (NZF-C) domain that recognizes ubiquitylated cargo (BOX 3). The GLUE domain of Vps36 has a pleckstrin homology (PH) domain fold (PDB ID 2CAY[56]) and facilitates interaction between ESCRT-II and phosphatidylinositol-3-phosphate (PtdIns3P)-containing endosomal membranes.

MIT
A domain found in microtubule interacting and trafficking proteins that forms a three-helix bundle. Some MIT domains, including those of VPS4 and AMSH, bind to CHMPs.

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with the core[83]. When released from autoinhibitory interactions, the MIR recruits MIT-domain-containing proteins such as the DUB AMSH (associated molecule with the Src-homology-3 (SH3) domain of STAM)[25,84,85] and the ATPase VPS4 (REF. 86), which disassembles the ESCRTs. The crystal structure probably represents the activated form of VPS24, with the helix α5 released from its autoinhibited conformation to make an arbitrary association with the core.

Oligomerization of ESCRT-III. Yeast two-hybrid, as well as biochemical approaches, have revealed a complex network of homo- and heteromeric interactions between ESCRT-III-related proteins[84,87–90]. It has been proposed that ESCRT-III proteins have a metastable, monomeric closed form in which the C-terminal region blocks heterodimerization with the cores of other ESCRT-III subunits until an activating influence displaces it[83,91]. The activated, opened-form heterodimers are thought to assemble into ESCRT-III lattices on endosomal membranes[76,87,88,90]. In the VPS24 crystal, dimers link together to form linear arrays via a second dimer interface that involves the tips of the α1–α2 hairpin (FIG. 4b). These arrays might be part of a continuous lattice on endosomal membranes[60], but it is not known whether these assemblies have a defined stoichiometry. It has been suggested that Vps2 and Vps24 form one subcomplex and Snf7 and Vps20 form another[76] (FIG. 4c). There is a pronounced sequence similarity of the subunits in each of the subcomplexes, enabling pairing in the subcomplexes that mimic homodimerization. Although the functional relevance of ESCRT-III lattices in the MVB pathway is not clear, it might be that they spatially restrict membrane-curvature-inducing factors to initiate budding away from the cytoplasm (FIG. 1). In yeast, the ENTH-domain-containing proteins Ent3 and Ent5, which interact with phosphoinositides and ESCRT-0, have been proposed as possible curvature inducers[92].

Interactions of ESCRT-III subunits with other MVB components. Having a closed, autoinhibited monomeric form is probably a general property of ESCRT-III subunits, as full-length ESCRT-III subunits do not appear to readily interact with each other in the cytosol. However, many interactions form upon recruitment to endosomal membranes, or upon expression of variants that are truncated or have large N- or C-terminal tags. Activation for assembly into lattices might be the consequence of triggering interactions with other components of the MVB pathway, such as ALIX (Bro1 in yeast)[4], the VPS25 subunit of ESCRT-II[62,63,66,76], membranes[93], AMSH[25,83,84,88,94] or ESCRT-III-related proteins such as CHMP1 (Did2 in yeast) (REFS 80,87,88,90) (FIG. 4C).

The VPS24 subunit has been reported to bind specifically to PtdIns(3,5)P_2 (REF. 93). An extensive, flat, basic surface in the structure of the VPS24 dimer, mostly contributed by helix α1, is likely to provide a membrane-binding interface (BOX 3). Mutation of basic residues in this helix reduces the dominant-negative effect of overexpression of VPS24 on viral budding and eliminates phosphoinositide binding *in vitro*[82,93]. Other ESCRT-III subunits have a less prominent basic surface, suggesting that a primary role of VPS24 might be to stabilize ESCRT-III on membranes. Additional membrane anchorage might be provided by myristoylation of VPS20 (REFS 66,76).

Importantly, multiple ESCRT-III-related proteins bind to the MIT domain of the AAA+ ATPase Vps4 (REFS 79,80,87,88,90,95) using their C-terminal MIT-interacting region[83,95] (FIG. 4). By engaging Vps4 with its C terminus and the Vps2 and Vps24 subunits with its N terminus, the ESCRT-III-related protein Did2 functions as an adaptor that is essential for the disassembly of ESCRT-III[80].

The role of ESCRT-III in the MVB pathway. In yeast, deletion of any ESCRT-III-related subunit causes an accumulation of endocytic cargo in class E compartments[79]. This, together with the observed dominant-negative effects of CHMP–green fluorescent protein (GFP) fusions in mammalian cells[66,81,96,97], was previously thought to imply that these proteins, like HRS (ESCRT-0) and TSG101 (ESCRT-I) are required for ILV formation. However, a number of recent studies indicate that ILV formation continues unabated in the absence of at least some of these proteins, whereas both endosomal morphology and cargo sorting to lysosomes are clearly affected.

Depletion of the ESCRT-III component VPS24 (also called CHMP3) in mammalian cells severely inhibits the degradation of EGFR[98], but the kinetics of signal termination remain unaltered[98]. A possible explanation for this finding is provided by ultrastructural analysis: in VPS24-depleted cells, activated EGFR is removed from the limiting endosomal membrane and sorted into the ILVs of the MVB[98], where it is incapable of transducing signals to cytosolic effectors. This contrasts distinctly with TSG101 depletion that inhibits ILV formation[15,54] and results in sustained EGFR signalling[98]. Similarly, homozygous knockout of *Chmp5* in mice is embryonic lethal and results in enlarged late endosomes that are densely packed with internal vesicles[99]. These apparently well developed MVBs are nevertheless incapable of degrading the fluid-phase marker horseradish peroxidase, TGFβIIR[99] or EGFR[96]. This indicates that VPS24 and CHMP5 are not essential for ILV formation but might have a role downstream in the pathway, possibly as a large multivalent recruitment platform for accessory proteins. Analysis of CHMP5-depleted cells indicates a defect in fusion between MVBs and lysosomes[99]. This might be due to a failure to recruit fusion-mediating components or a failure to dissociate the ESCRT-III lattice.

Deletion of the peripheral ESCRT-III-related Did2 protein in yeast likewise does not block ILV formation, but prevents efficient incorporation of cargoes into the ILVs[80]. Did2 seems to function as an adaptor that couples Vps4 to dissociation of at least some ESCRT-III, but not ESCRT-I and –II, subunits from endosomes. These data indicate that ILV formation can be uncoupled both from cargo sorting and ESCRT-III disassembly.

AAA + ATPases
ATPases associated with various cellular activities: a superfamily of structurally related proteins that control diverse functions, including protein disassembly.

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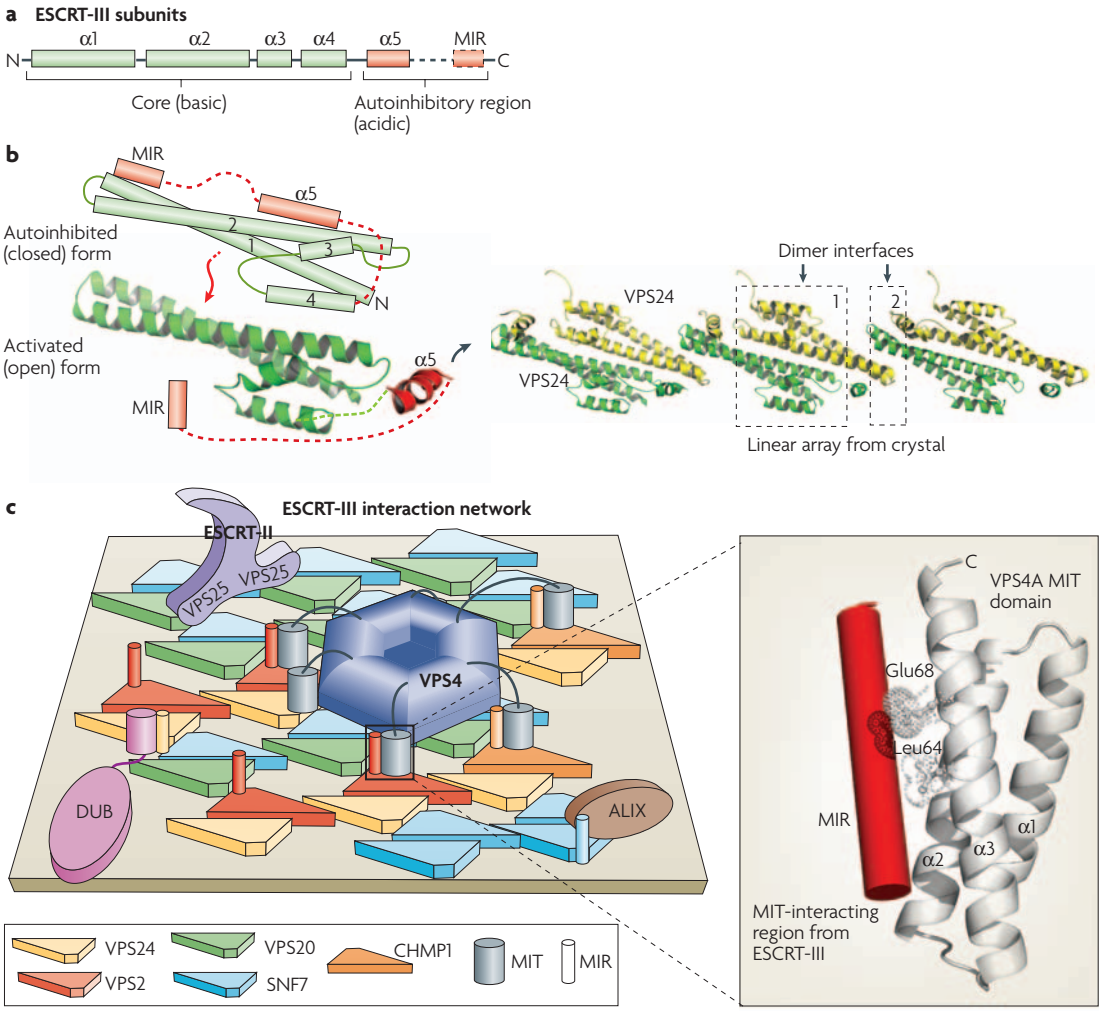

Figure 4 | **The architectures of ESCRT-III. a** | The ESCRT-III subunits have related sequences with prominent bipolar character, and probably similar helical organizations to VPS24. **b** | ESCRT-III subunits can probably adopt an autoinhibited (closed) monomeric state. A conformational change to the activated (open) form that relieves the autoinhibition conferred by the C-terminal region is suggested (left panel). The structure of a VPS24 dimer in a linear array from the crystal (Protein Data Bank (PDB) ID 2GD5 (REF. 82)) suggests how activated conformations might assemble into lattices on membranes (right panel). Both dimer interfaces are important for the function of VPS24. **c** | Proposed formation of an ESCRT-III lattice on endosomal membranes. Each ESCRT-III subunit has a C-terminal autoinhibitory region. Displacement of the autoinhibitory region of VPS20 through interactions with the VPS25 subunit of ESCRT-II might trigger assembly of the lattice. In the activated conformation, the C-terminal region can interact with the microtubule-interacting and transport (MIT) domains from VPS4 and (in mammals) the de-ubiquitinase (DUB) AMSH (associated molecule with the Src-homology-3 domain of STAM; pink). The inset shows the structure of the MIT domain of VPS4A (PDB ID 1YXR[95]) and highlights Leu64 and Glu68, which are important for interaction with CHMP1B. The interaction with a putative C-terminal helix (MIT-interacting region (MIR)) from an ESCRT-III subunit is also modelled in the inset. A VPS4 complex is illustrated as a hexagon. The central pore might have a role in taking up and releasing ESCRT-III subunits that are disassembled from the lattice. CHMP1 (orthologue of yeast Did2) is pictured in its role as an adaptor that interacts with ESCRT-III core subunits and VPS4.

Dissociation of ESCRT-III

VPS4-dependent disassembly of the ESCRT components is required for sorting into MVBs. In yeast, all of the ESCRTs remain tightly associated with membranes in the absence of Vps4 (REFS 76,80,100). In mammalian cells, VPS4 function is required to disassemble the clathrin bilayer coats in which cargo is clustered[101] and in both yeast and mammalian cells it is required for the formation of ILVs.

The role of VPS4 in the MVB pathway. In humans there are two isoforms of VPS4 (A and B) that are 80% identical. VPS4B can functionally complement the 60% identical yeast Vps4 (REF. 102). These enzymes belong to a subset of AAA+ ATPases, typically hexameric enzymes, with the ATP-binding site located at the intersubunit interfaces.

In yeast, loss of Vps4 results in accumulation of ESCRT components on membranes, impairment of cargo sorting and a class E phenotype[103]. Dissociation

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of ESCRTs requires the ATPase activity of Vps4. In mammalian cells, a mutation in VPS4 that prevents ATP hydrolysis, but not ATP binding, has a dominant-negative phenotype both in viral budding and in the MVB pathway, where it promotes accumulation of ESCRTs on enlarged late endosomes and delays the formation of internal vesicles[51,87,101,104]. In analogy with the function of other AAA+ type ATPases to disassemble complexes such as SNARES and microtubules, it is possible that Vps4 disassembles ESCRT networks by partially unfolding subunits, thereby disrupting key interactions that maintain the integrity of an ESCRT lattice assembled on membranes.

Structure of Vps4. The crystal structure of the human VPS4B has been determined in a monomeric, nucleotide-free form[86]. In the presence of ATP, VPS4 assembles into a complex that contains 10–12 subunits. On the basis of comparisons with existing AAA+ ATPase structures, it has been proposed that the oligomeric VPS4 consists of two hexameric rings stacked on each other.

At the centre of the VPS4B hexameric ring model is a hydrophobic pore[86]. Mutations of residues in this pore do not affect the assembly of VPS4 oligomers, but impair HIV-1 release and infectivity. This suggests that ESCRT-III lattices might be disassembled by being drawn into the central cavity of the VPS4 oligomer via this pore[86], in analogy to the proposed mechanism of substrate entry for the p97 AAA+ ATPase[105]. The constricted channel and pore suggest that partial unfolding of the ESCRT-III substrate might be required. It is not clear how an ESCRT-III subunit would be released once it is drawn into the cavity, nor whether and how this might be coupled to budding of cargo-carrying ILVs.

Interaction of VPS4 with other MVB components. VPS4 is recruited to endosomal membranes through an interaction between its N-terminal MIT domain (a three-helix bundle) and the C-terminal portion of ESCRT-III-related proteins[76,88,91,95] (FIG. 4). Oligomerization of VPS4 is facilitated by ATP binding; however, there is growing evidence that in cells this is catalysed by association with the protein Vta1 (LIP5 in mammals)[86,100,106,107], which increases the rate of ATP hydrolysis. Vta1 deletion produces delayed vacuolar trafficking in yeast[100]. Depletion of the mammalian homologue, LIP5, impairs HIV-1 infectivity and slows degradation of internalized EGFR without preventing ILV formation or affecting endosomal morphology[96]. In addition, LIP5 (Vta1) also interacts with the ESCRT-III related protein, CHMP5 (REFS 96,100), and this interaction does not compete with VPS4 (REF. 100). In yeast, deletion of the CHMP5-orthologue Vps60 causes prolonged retention of Vta1 in a class E compartment, suggesting that Vps60 might have a role in recycling Vta1 (REFS 100,108).

De-ubiquitylation in the ESCRT pathway

De-ubiquitylation of cargo precedes its incorporation into an ILV and serves to maintain levels of free ubiquitin. DUBs might also be recruited early in the pathway, where they could provide a proof-reading mechanism and rescue cargo before commitment to the degradative pathway. Many proteins that are involved in endocytic cargo sorting are ubiquitylated, and it has been proposed that another role of de-ubiquitylation might be to directly regulate the ESCRT machinery[109,110].

Doa4: a DUB for the MVB pathway in yeast. There are 16 DUBs in yeast, but until recently only degradation of alpha-4 (Doa4) had been implicated in the MVB pathway[110–112]. Doa4 function is required for efficient traffic to the vacuole[45,77,112,113]. However, modified chimeric cargo that has ubiquitin fused to its C terminus so that it cannot be removed by DUBs is trafficked to the vacuole, even in the absence of Doa4, indicating that de-ubiquitylation is not a prerequisite for cargo sorting[44,112]. Depletion of free ubiquitin levels in cells might indirectly affect the sorting process in *doa4*-mutant yeast, as overexpression of ubiquitin can partially rescue some of these defects[111,112]. Efficient de-ubiquitylation requires Bro1, which has an important role in recruiting Doa4 to endosomes[109,114] (reviewed recently elsewhere[4]), although a direct interaction between Doa4 and Snf7 has also been reported[89].

Doa4 is a Cys protease of the ubiquitin-specific protease family (UBP). The structures of the catalytic cores of two mammalian members of the UBP family, UBPY (also known as USP8)[115] and HAUSP[116], show three domains that are referred to as the thumb, palm and fingers. Ubiquitin rests on the palm domain and interacts with both the finger and thumb. The C terminus of ubiquitin threads into the active site of the DUB so that the enzyme can cleave the isopeptide bond that links ubiquitin and the cargo. The surface patch of ubiquitin that includes Ile44 interacts with the DUB, precluding simultaneous interaction with any other ubiquitin-binding proteins of the ESCRT pathway. Consequently, ubiquitin binding by an MVB-associated DUB represents the final, vectorial recognition of ubiquitylated cargo.

Mammalian DUBs associated with the ESCRT machinery. On the basis of sequence and domain conservation, UBPY is the most likely Doa4 orthologue in mammalian cells. UBPY interacts with the SH3-domain of the ESCRT-0 component STAM via two non-canonical binding motifs[117,118], one of which is also conserved in the JAMM-domain DUB AMSH, which is activated by association with STAM[25,117]. Similarly, the yeast orthologue of STAM, Hse1, also associates both directly and indirectly via its SH3-domain with the DUBs Ubp7 and Ubp2, respectively[119]. These interactions might be crucial to recruiting DUBs early in the pathway, where they might promote disengagement of the cargo from the ESCRT machinery[25,119]. Like the two members of ESCRT-0, AMSH binds to clathrin, but in addition, its N-terminal region (residues 1–127) has a MIT-like domain that binds multiple CHMPs[25,83,84,88,94]. AMSH depletion does not impair but rather enhances the kinetics of EGF and EGFR degradation[75,85,94]. A recent study

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Proteasome
Multi-enzyme complex responsible for the majority of protein turnover in eukaryotic cells that degrades proteins tagged with a particular type of ubiquitin chain.

reports an accumulation of ubiquitylated proteins on and in endosomes upon AMSH depletion, indicating that de-ubiquitylation and cargo degradation can be uncoupled in these cells[94].

In contrast to AMSH and Ubp7, UBPY and Ubp2 activity appear to be required for progress along the MVB pathway[75,119–123]. UBPY-depleted cells fail to degrade multiple activated receptor Tyr kinases (RTKs), including EGFR, which accumulate on endosomes in their ubiquitylated form[75,120–122]. These cells also show an increase in the number and size of MVBs, which are aberrantly attached to one another and to lysosomes via tethers[120,121], suggesting a defect in MVB–lysosome fusion. The effect on receptor degradation might be partially explained by a severe loss of STAM, which is polyubiquitylated and degraded by the proteasome in the absence of UBPY[120]. The key target of Ubp2 activity is currently unclear[119], but in contrast to UBPY, and in common with AMSH, it shows preferential activity towards Lys63-linked ubiquitin chains, which have been implicated in endocytic trafficking[25,85,113,123].

Conclusions

The players in the MVB pathway have largely been identified, and structural work has provided a solid understanding of how the complexes are assembled, how they interact with each other and how they engage with the central sorting signal, ubiquitin. Great progress has been made in elucidating the molecular basis for lipid recognition by many of the endosomal and MVB components, but new players with new tricks can be expected. Several key questions remain unanswered. What is the molecular organization of the ESCRT-III lattice? What is its function? How is it disassembled? What are the roles of the multiple isoforms of mammalian ESCRT subunits? Are any of them functionally redundant? The major challenge for the future is to define the mechanisms of internal vesicle budding and of even less studied processes such as back fusion of ILVs with the lumenal membrane. This will require bridging the gap between atomic-resolution three-dimensional (3D) structures provided by NMR and crystallography, and the nanometer resolution of the breathtaking 3D EM tomographic reconstructions of endosomes.

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Competing interests statement
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FURTHER INFORMATION
Roger L. William's homepage:
http://www2.mrc-lmb.cam.ac.uk/PNAC/Williams_R
Sylvie Urbé's homepage:
http://www.liv.ac.uk/~urbe/SU-webpage.html
Access to this links box is available online.

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