Membrane Protein Targeting to the MVB/Lysosome

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Received September 18, 2008

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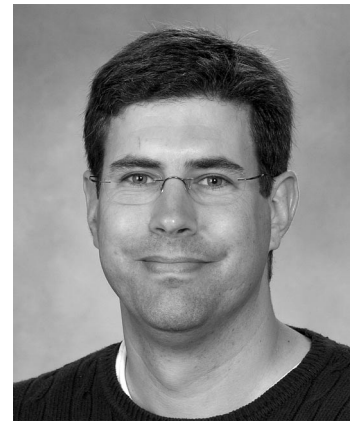

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1. Introduction: MVB Sorting Terminates Protein Function

Cells sense and respond to their environment largely through the function of receptors, transporters, and channels within the plasma membrane. For cells to appropriately respond to environmental cues, they must maintain the proper protein complement at the cell surface. This involves both delivering proteins to the cell surface and removing them when necessary. The multivesicular body (MVB) sorting reaction within the endocytic pathway provides an important cellular mechanism for terminating the function of integral membrane proteins destined for degradation within the lysosome (reviewed in refs 1−3). The MVB sorting machinery recognizes a subset of endocytic cargoes and concentrates them into regions within the endosomal membrane. These sections then bud as small vesicles into the lumen of the endosome, giving the endosome a multivesicular appearance by electron microscopy. Subsequent fusion of the MVB with the lysosome delivers these intralumenal vesicles to the hydrolytic environment of the lysosome, where the lipid and protein contents of the vesicles are degraded (reviewed in ref 4). The topology of membrane invagination into the endosomal lumen (exvagination from the cytosol) during MVB sorting is similar to the budding process whereby enveloped viruses, including HIV-1 and Ebola virus, egress from the cell (Figure 1). Viral structural proteins, such as Gag from HIV-1 and VP40 from Ebola virus, utilize the MVB sorting machinery to bud either from the plasma membrane or into an intracellular compartment for subsequent release from the cell (reviewed in refs 5 and 6). Therefore, the MVB sorting machinery is important both for viral replication and for lysosomal delivery of endocytosed proteins. In addition, a number of recent studies have shown a role for the MVB sorting machinery in late steps of cytokinesis,[7−13] emphasizing the importance of this machinery for diverse cellular processes of similar membrane topology.

MVB sorting is conserved throughout eukaryotes, and studies in both yeast and mammalian systems have identified a series of *trans*-acting factors that mediate this reaction (reviewed in refs 2, 14, and 15). The endosomal sorting complexes required for transport (ESCRTs) and associated proteins constitute the majority of this machinery. Cargo recognition is mediated by interactions with the Vps27/Hrs-Hse1/STAM complex as well as with ESCRT-I (Vps23/Tsg101, Vps28, Vps37, and Mvb12).[16−20] ESCRT-II (Vps22, Vps25, Vps36) appears to function downstream or in parallel to ESCRT-I and is also believed to interact with cargo based on ubiquitin-binding domain activity within Vps36.[21−25] ESCRT-II additionally serves to facilitate the recruitment and assembly of ESCRT-III subunits (Vps20/CHMP6, Snf7/CHMP4, Vps2/CHMP2, Vps24/CHMP3, Did2/CHMP1,

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10.1021/cr800473s CCC: $71.50
Published on Web 02/25/2009

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Vps60/CHMP5) into a polymer.[25−28] Interactions between ESCRT-III and the Vps4-Vta1 complex then promote disassembly of ESCRT-III and may be linked to the completion of intralumenal vesicle (ILV) formation.[29−35] The concerted actions of these multimeric complexes serve to concentrate and deliver MVB cargoes into ILVs of the endosome for their eventual degradation within the lysosome. While lysosomal degradation provides a mechanism for the long-term attenuation of transmembrane proteins (such as cell surface receptors, transporters, and channels), sorting into the MVB appears to abrogate their functions prior to degradation. Sorting activated receptors into the MVB sequesters their cytoplasmic kinase domains into the endosomal lumen and away from cytoplasmic substrates, effectively terminating signaling from activated receptors. A well-studied example illustrating the importance of this reaction is the sorting of activated epidermal growth factor receptor (EGFR) (reviewed in refs 36 and 37). Defects that prevent EGFR sorting into intralumenal vesicles are oncogenic due to receptor hyperactivity, and this oncogenic activity is attributed to deficiencies in both receptor sequestration and lysosomal degradation (reviewed in refs 38 and 39). While MVB sorting can have an important impact on

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signaling, signaling can also impact MVB sorting. EGFR delivery into the MVB pathway is accelerated in response to EGF stimulation; moreover, EGF stimulation actually promotes MVB biogenesis.[40,41] The Hrs-STAM complex is phosphorylated after stimulation of receptor tyrosine kinases,[42−44] and this phosphorylation impacts machinery levels and receptor degradation.[45] Therefore, it is important to understand the mechanisms by which signaling and MVB sorting impact each other and how flux through the MVB pathway is regulated.

2. Cargo Recognition

2.1. Sorting Determinants within MVB Cargoes

The delivery of proteins into the MVB pathway is an active process that is tightly regulated to maintain the proper cellular protein complement. The predominant signal driving entry into this pathway is the covalent modification of the cargo with ubiquitin (reviewed in refs 2 and 46−48). Early studies of endocytic cargoes demonstrated a role for ubiquitin modification during endocytosis and lysosomal targeting in both yeast and mammals but did not afford the resolution to identify the stages at which this post-translational modification was contributing to receptor downregulation.[49−51] Lysosomal trafficking of EGFR in mammalian cell lines had been demonstrated to include entry into a MVB,[52] and lysosomal trafficking was disrupted by dysfunction of the ubiquitin ligase c-Cbl.[53] Perturbation of c-Cbl function resulted in decreased EGFR ubiquitination and enhanced EGFR signaling;[53−55] however, a specific defect in EGFR MVB sorting was not appreciated because of contributions of ubiquitination to receptor internalization as well. Several studies utilizing cargoes that do not transit the cell surface (biosynthetic MVB cargoes and ubiquitin chimeras) provided evidence that ubiquitin modification was playing an essential role in targeting MVB cargoes into ILVs.[18,56,57] Coincidently, a number of ubiquitin-binding domains (UBDs) were identified within components of the MVB sorting machinery, immediately suggesting a mechanism by which these ubiquitinated cargoes are recognized.[16−19] These observations were further supported by the characterization of mutant forms of the ubiquitin ligase Rsp5 in *Saccharomyces cerevisiae* that were defective for the targeting of ubiquitin-

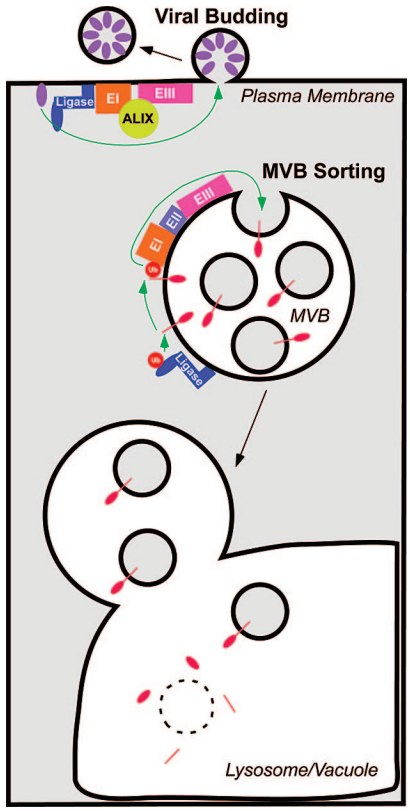

Figure 1. Roles for cellular machinery in MVB sorting and viral particle formation. Ubiquitin ligases, ESCRTs, and associated factors (such as Alix, shown, or Vps4/SKD1, not shown) have been demonstrated to execute critical functions during MVB sorting (below) and viral budding (above). Vesicle formation is topologically similar in both cases because deformation is away from the cytoplasm. Viral budding can occur from the cell surface or into an intracellular compartment, but only the former is presented for simplicity. In both contexts, ubiquitin ligases can play a critical role in cargo selection through covalent modification of cargoes as well as an adaptor role independent of cargo ubiquitination. The role of Alix/Bro1 in MVB sorting is less clear than in viral budding, where it appears to play a role in cargo selection via bridging late domains with the ESCRTs (in which ESCRT-I and -II may be bypassed). EI, = ESCRT-I; E-II, ESCRT-II; EIII, ESCRT-III; Ligase, ubiquitin ligases involved in these sorting events; Ub, ubiquitin. Purple ovals represent viral structural protein (e.g., Gag); red ovals with stalks represent MVB cargo protein.

dependent MVB cargoes without gross defects in MVB function.[58] These analyses demonstrated a role for ubiquitin in cargo sorting into the MVB pathway in yeast. Ubiquitination has subsequently been established as a critical MVB sorting determinant for a large number of cargoes throughout eukaryotes.

2.1.1. Ubiquitination as a Sorting Signal

Ubiquitin is a versatile post-translational modification. Initially, polyubiquitination was appreciated as a signal for degradation by the proteasome (reviewed in ref 59). However, by the late 1990s, additional functions had been attributed to ubiquitination, including transcriptional regulation, kinase activation, mitochondrial inheritance, DNA repair, and intracellular protein sorting (reviewed in refs 60 and 61). Ubiquitination is a reversible modification and has been compared to phosphorylation in terms of its diverse regulatory outcomes. Whereas phosphorylation is a small molecular modification of a substrate, ubiquitin is itself a ~8.5 kDa protein that is conjugated to substrates via an isopeptide linkage. This distinction affords a number of unique characteristics to ubiquitination as a post-translational modification.

Whereas phosphopeptide recognizing domains distinguish the phosphorylated residue in a particular substrate or sequence context, most ubiquitin binding domains interact with a surface surrounding Isoleucine 44 of ubiquitin that is actually distal to the linkage between ubiquitin and the substrate (ref 62 and reviewed in refs 2, 47, and 63). This suggests that, once a substrate is modified with ubiquitin, ubiquitin itself serves as the predominant interaction determinant with effector proteins. This feature offers the potential advantage of permitting MVB cargoes to be ubiquitinated at a number of sites, thereby allowing a broad spectrum of proteins to be recognized by the MVB sorting machinery once they have received this modification. Interestingly, the affinities of the ubiquitin binding domains for ubiquitin appear to be fairly weak (in the micromolar range).[63] This low affinity in combination with the observation that multiple components of the MVB machinery harbor UBDs suggests that multiple transient interactions between ubiquitinated cargoes and the MVB machinery facilitate entry into the pathway.

A second feature unique to ubiquitin as a post-translational modification is its ability to be conjugated to itself, resulting in polyubiquitination (reviewed in ref 64). Covalent linkage with ubiquitin occurs via primary amines, usually a lysine residue but sometimes the amino terminus of a protein.[65] Because ubiquitin itself contains lysine residues, covalently linked ubiquitin can itself be modified with ubiquitin to give rise to polyubiquitin chains. Ubiquitin has 7 lysine residues, all of which have been shown to be acceptor sites for ubiquitination in yeast.[66] Substrates with polyubiquitin chains linked at lysine 48 or lysine 63 appear to have distinct fates in vivo. Lysine 48-linked chains are predominantly associated with proteasomal degradation, a fate typically reserved for soluble proteins or misfolded membrane proteins recognized by the ERAD pathway.[67−70] Lysine 63-linked chains are associated with trafficking and signaling (ref 71 and reviewed in ref 61). Mass spectroscopy analysis has also shown that a single ubiquitin molecule can be modified at multiple residues, forming chains of mixed topology.[72] Whether these mixed chains have functional significance with respect to trafficking is not yet clear. The specific linkages within these polyubiquitin chains may have important consequences for MVB sorting or other activities.

The most favorable ubiquitin configuration to promote MVB sorting is still under investigation. While some MVB cargoes are polyubiquitinated, monoubiquitination is sufficient to promote yeast MVB sorting as ubiquitin-MVB cargo chimeras enter the pathway in strains defective for the ubiquitin ligase Rsp5, as can ubiquitin-MVB cargo chimeras that cannot undergo polyubiquitination.[57,58,73,74] In mammals, EGFR has been shown to be monoubiquitinated at multiple lysine residues (multiubiquitinated) in vivo, and multiubiquitinated forms of EGFR are quickly degraded in the lysosome;[75] however, a recent report on EGFR sorting indicates that polyubiquitination, as well as multiubiquitination, contributes to efficient entry of EGFR into the MVB pathway.[76,77] A recent analysis using CD4-based chimeras simulating monoubiquitinated cargo, multiubiquitinated cargo, or polyubiquitinated cargo found that multiubiquitination or polyubiquitination through K63-linked chains promotes robust delivery to the lysosome.[78] K48-linked polyubiquitin

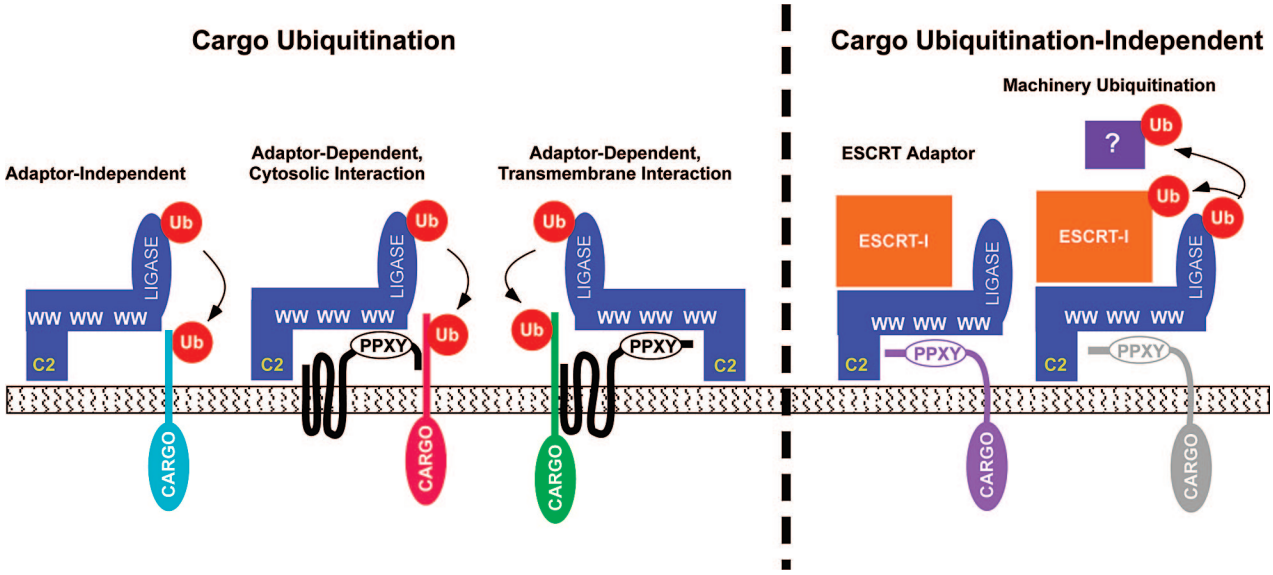

Figure 2. Potential roles for ubiquitin ligase function during MVB cargo selection. In this model, cargo selection is considered as both MVB sorting and viral budding. Membrane association of ligase may be facilitated by lipid binding domains such as the C2 domain found in Nedd4 homologues. Membrane association may also be impacted by membrane adaptors (represented by the black PPXY-containing membrane protein), which may also impact cargo ubiquitination. Ligase recruitment to cargo may also occur via WW-PPXY interactions, which may additionally impact cargo or machinery ubiquitination during MVB sorting. These roles for ligases during cargo selection are not mutually exclusive. For example, machinery ubiquitination may be required to facilitate MVB sorting regardless of cargo ubiquitination status.

chains can also lead to lysosomal delivery, but less efficiently than K63-linked chains.[78] One possible interpretation of these results is that the location of ubiquitination (i.e., at an endosomal compartment wherein the ubiquitinated cargo can be recognized) may be more critical than the specific ubiquitin linkage. While it may be difficult to conclusively identify the ubiquitin configuration optimal for MVB sorting given the dynamic, reversible nature of ubiquitination, this issue continues to be under examination.

2.1.2. Sorting Signals Independent of Cargo Ubiquitination

While ubiquitination serves as the predominant sorting signal, some cargoes do not require ubiquitination of themselves to enter the MVB pathway. One such example of cargo ubiquitination-independent sorting is the yeast protein Sna3.[56,79−81] While Sna3 is subject to ubiquitination by the ubiquitin ligase Rsp5, this modification is not required for its MVB sorting.[66,79−81] GFP-Sna3 with all lysine residues converted to arginines is defective for ubiquitination yet still enters the MVB pathway; however, lack of Sna3 ubiquitination reduces the kinetics of this sorting.[80] These results demonstrate that ubiquitination can potentiate, but is not required for, Sna3 MVB sorting. Examination of the Sna3 cytoplasmic portions for alternative sorting determinants identified a tyrosine-containing motif in the amino-terminus and a PPXY motif in the carboxy-terminus.[79−82] While the mechanism of tyrosine motif-dependent sorting remains unclear, examination of the PPXY motif yielded a surprising result. The ubiquitin ligase Rsp5 was demonstrated to interact with Sna3 via this motif and the WW domains of Rsp5. While Sna3 ubiquitination is not required, the Sna3-Rsp5 interaction and Rsp5 ligase activity are required for Sna3 MVB sorting.[79−81] These observations suggest that Rsp5 may bind Sna3 to link Sna3 to other components of the MVB sorting machinery or that Rsp5 associated with Sna3 may ubiquitinate components of the MVB sorting machinery to promote Sna3 sorting (Figure 2); in addition, the association between Rsp5 and Sna3 promotes ubiquitination of Sna3, which also potentiates its sorting into the MVB pathway. These yeast studies highlight a potential mechanism by which a ubiquitin ligase can promote sorting of a MVB cargo without requiring ubiquitination of the cargo itself.

Similarly, viral proteins may not need direct ubiquitination but may still require ubiquitin ligase activity for budding. Enveloped viruses can usurp the MVB sorting machinery in mammalian cells to allow budding at either the plasma membrane or into intracellular compartments for release (reviewed in ref 5). While ubiquitination appears to promote the budding of viral structural proteins such as HIV-1 Gag, the roles of ubiquitination and ubiquitin ligases in viral budding are not fully understood (reviewed in ref 6). However, the prototypic foamy virus (PFV) provides a compelling model for cargo ubiquitination-independent viral budding in human cells. A PFV Gag protein modified through mutation of the ubiquitin-acceptor lysine residues retains the ability to form viral-like particles (VLPs).[83] PFV Gag contains a PSAP late domain that is required for VLP production; this late domain binds to the ESCRT-I subunit Tsg101, the mammalian homologue of Vps23.[84,85] While mutation of the PSAP motif abrogates this ubiquitination-independent PFV Gag VLP formation, swapping the PSAP motif with a PPXY motif recruits the Nedd4/Rsp5-family ubiquitin ligase WW1 to the plasma membrane and enables VLP budding.[83] This suggests that recruitment of a Nedd4/Rsp5 family ligase can permit viral budding and that ubiquitin ligases can serve as a link to engage the mammalian MVB machinery without direct cargo ubiquitination (Figure 2).

While the yeast cargo protein Sna3 and viral structural proteins appear to interact with the MVB sorting machinery via their cytoplasmic sorting determinants, a subset of cargoes enter the MVB pathway independent of their cytoplasmic domains. Ath1 is sorted into the yeast vacuole where it degrades trehalose, a sugar produced during cellular stress.[86] Delivery of Ath1 to the vacuolar lumen occurs via

MVB sorting and depends on the MVB sorting machinery. However, in contrast to the majority of characterized MVB cargoes, sorting of Ath1 does not require its cytoplasmic domain.[87] Further examination suggested that the Ath1 transmembrane domain harbors a novel MVB sorting determinant, although the mechanism driving recognition of this motif is still unclear. These results expanded on insights gained from analyses of other model MVB cargoes (e.g., CPS and Sna3) to demonstrate that both the cytoplasmic and transmembrane domains can harbor MVB sorting determinants in yeast. One possible candidate for recognition of MVB cargo transmembrane domains is the Rsp5-interacting protein Bsd2.[88] Alternatively, sequestration into lipid microdomains could facilitate sorting of transmembrane-encoded MVB sorting motifs.

In mammalian cells, the determinant for Pmel17 sorting into melanosomes resides in neither the cytoplasmic nor transmembrane domains. Melanosomes are lysosome-like organelles found in melanocytes in the skin as well as pigment cells of the eyes (reviewed in ref 89), and delivery of Pmel17 into the melanosome occurs via an MVB-like intermediate.[90] Consistent with this similarity, ectopic expression of Pmel17 in nonmelanocytes leads to its delivery into MVBs and eventually the lysosome.[91] Pmel17 is a transmembrane protein that is sorted into the intralumenal vesicles (ILVs) of early endosomes and is then diverted into melanosomes. The delivery of Pmel17 into this pathway does not require Pmel17 ubiquitination. Instead, the lumenal portion of Pmel17 is sufficient to confer proper targeting, and Polycystic Kidney Disease 1-like (PKD) repeats constitute the sorting determinant within the lumenal domain.[92] One possibility is that the PKD repeats permit association with another (conventional) ILV/MVB cargo. However, the common MVB sorting machinery appears to be dispensable for Pmel17 sorting, as disruption of MVB sorting via the overexpression of Hrs, Tsg101, or $Vps4^{K173A}$ or the depletion of Hrs did not perturb Pmel17 distribution.[92] While the mechanism by which the sorting motif determines trafficking remains unclear, Pmel17 illustrates that the determinant for MVB sorting can also reside within the lumenal domain. Overall, these observations indicate that there are multiple methods for proteins to enter the MVB pathway—both through the predominant mechanism of cargo ubiquitination and via alternative sorting motifs that can be located in the cytoplasmic, transmembrane, or lumenal domains.

2.2. Factors Regulating Cargo Ubiquitination

To date, ubiquitination is the predominant signal for cargo inclusion into the MVB pathway. Regulation of ubiquitination status thus constitutes a critical determinant for MVB sorting of a cargo. Both the ubiquitin ligase complexes and deubiquitinating complexes serve roles in maintaining the proper cargo ubiquitination status, and recent studies have provided insights suggesting that these activities can be coordinated.

Ubiquitin modification of cargo proteins occurs through the concerted action of the ubiquitin activating enzyme (E1), the ubiquitin conjugating enzyme (E2), and the ubiquitin ligase (E3) (reviewed in ref 59). Of these, the E3 enzymes are the most diverse and confer specificity to the ubiquitination reaction through combining lipid- and protein-interaction motifs with catalytic ubiquitin ligase domains. The predominant ubiquitin ligase domains identified contain either HECT (homologous to E6 C-terminus) or RING (really interesting new gene) domains (reviewed in refs 93−95). In yeast, five known components of the endocytic system harbor RING domains: Vps8, Vps11, Vps18, Pib1, and Tul1. While Tul1 appears to contribute to MVB sorting in some contexts,[58,73,74,88] a role for ubiquitination by these other RING ligases in MVB sorting has not been demonstrated. Instead, the Nedd4-family ligase Rsp5 represents the major ubiquitin ligase activity within the MVB sorting system.[58,74] Rsp5 harbors a C2 domain and three WW domains in addition to a HECT domain. While the C2 domain has been implicated in endosomal localization via lipid binding,[96] the role of the WW domains may be multifaceted, contributing to ubiquitination of specific MVB cargoes or adaptor proteins through binding PPXY motifs (Figure 2). For cargoes such as Sna3, this appears to occur through Rsp5 binding a PPXY motif within the cargo itself.[79−82] Nedd4 ubiquitination of the human epithelial sodium channel (ENaC) also appears to occur through recognition of a PPXY motif in the ENaC cytoplasmic tail.[97] However, other cargoes, including CPS, do not contain a conspicuous Rsp5-binding motif. Instead, adaptor proteins, including Bsd2, Ear1, and Ssh4, appear to bridge the interaction to facilitate cargo ubiquitination.

Ear1 and Ssh4 are two redundant proteins that contribute to the sorting of a subset of MVB cargoes, including the general amino acid permease Gap1, the uracil transporter Fur4, the iron/siderophore transporter Sit1, and the vacuolar polyphosphatase Phm5.[98] Fusion of ubiquitin to Phm5 (Ub-Phm5) bypasses the requirement for Ear1 and Ssh4 in Phm5 MVB sorting, implicating these two factors in cargo ubiquitination. This role was supported by the observation that loss of both Ear1 and Ssh4 abrogates Sit1 ubiquitination similar to the defect observed in an *rsp5* mutant. The contributions of Ear1 and Ssh4 to cargo ubiquitination occur in a cargo-specific manner as Sna3, a cargo that can directly interact with Rsp5 (discussed above), and Smf1 (discussed below) still undergo MVB sorting in *ear1*Δ *ssh1*Δ cells. While PPXY motifs in the cytoplasmic domains of Ear1 and Ssh2 likely mediate Rsp5 association, it remains unclear how these proteins promote ubiquitination of a particular subset of MVB cargoes.

By contrast, the transmembrane domain of the Rsp5 adaptor Bsd2 appears to permit direct association with another set of MVB cargoes, including CPS, to facilitate cargo ubiquitination. Bsd2 and CPS interact via their transmembrane portions while Bsd2 contains cytoplasmic PPXY motifs that recruit Rsp5[88] (Figure 2). Bsd2 acts in a similar manner for MVB sorting of the metal transporter Smf1, with the additional assistance of Tre1 (or the closely related Tre2).[99] In the case of Smf1 MVB sorting, Bsd2 and Tre1 appear to associate within the membrane while Tre1 and the MVB cargo Smf1 interact via cytoplasmic determinants. Rsp5 in turn binds both Bsd2 and Tre1 with the additional complication that WW domains (specifically WW2 and WW3) are implicated in combinatorial binding of the PPXY motifs in both Bsd2 and Tre1. These Rsp5 interactions with Bsd2 and Tre1 then allow ubiquitination of Smf1 and subsequent MVB sorting.[99] These examples from yeast illustrate both the importance of adaptors in Rsp5 cargo ubiquitination and the potential contribution of multiple WW domains within Rsp5 to this process. The presence of mammalian Bsd2 homologues (N4WBP5 and N4WBP5A) suggests that adaptors facilitate ubiquitination by the Nedd4 family in mammalian systems as well.[100]

The ubiquitination of cargoes for sorting into the MVB appears to be a dynamic process impacted by both ubiquitin ligases and deubiquitinating enzymes (DUBs). While the role of ubiquitin ligases is appreciated, the contributions of DUBs are less well understood (reviewed in ref 101). Many direct and indirect roles have been proposed for DUBs in MVB sorting, including recycling ubiquitin to maintain a cellular ubiquitin pool, trimming cargo-linked ubiquitin chains to favorable configurations, and allowing deubiquitinated cargo to exit from the MVB sorting reaction in response to cellular signals. Although some of these functions have yet to be examined directly, recent studies in yeast and mammalian cell lines indicate a complex role for DUB activity in MVB sorting and suggest that ubiquitination and deubiquitination activities are coordinated to a larger extent than previously appreciated.

Yeast Doa4 was the first DUB to be implicated in MVB cargo sorting. Loss of Doa4 results in a depletion of cellular ubiquitin levels, pointing to a role for Doa4 in ubiquitin recycling subsequent to cargo selection in the MVB pathway but prior to vesicle formation.[18,56,102−104] Consistent with this model, Doa4 is associated with Bro1, which also binds ESCRT-III, a late-acting component of MVB sorting.[105−108] In addition to facilitating Doa4 recruitment, Bro1 stimulates Doa4 catalytic activity through an interaction between a C-terminal motif in Bro1 and the catalytic domain of Doa4; defects in either protein that disrupts this activation lead to impaired cargo deubiquitination and defects in MVB sorting in vivo.[109] Doa4 overexpression suppressed the defects in endosomal morphology and cargo sorting found in cells lacking Bro1,[107] suggesting that Doa4-mediated maintenance of the free ubiquitin pool is the critical function mediated by the Doa4-Bro1 complex. However, restoring free ubiquitin levels in a *doa4* mutant by overexpressing ubiquitin does not rescue MVB sorting of CPS or Gap1, two ubiquitin-dependent MVB cargos.[110] This observation suggests that Doa4 may contribute an additional function beyond maintaining levels of free ubiquitin. Recently, Doa4-Bro1 has been found to associate with the ESCRT-I complex via Vps23, raising the possibility of an earlier function for Doa4 than previously appreciated.[108] In addition, this study also found that the ubiquitin ligase Rsp5 also associates with Bro1. While cargo ubiquitination has been anticipated to be a dynamic process, the identification of a single factor (Bro1) linking these activities of cargo ubiquitination (Rsp5), cargo deubiquitination (Doa4), and cargo recognition (ESCRT-I) was unexpected. While the mechanisms coordinating this dynamic process are still unclear, these studies highlight the possibility of additional functions for Doa4 in regulating ubiquitin-mediated cargo recognition in addition to contributing to ubiquitin recycling later during the MVB sorting pathway.

Two additional deubiquitinating enzymes, Ubp2 and Ubp7, have been implicated in MVB cargo selection in yeast.[111] Ubp2 associates with Rsp5, and loss of Ubp2 results in the accumulation of K63-linked ubiquitin chains.[112,113] The Rsp5-Ubp2 complex and Ubp7 associate with the MVB sorting machinery through Hse1, a protein associated with the early-acting cargo recognition factor Vps27.[111] While cells lacking Ubp2 exhibit impaired ubiquitin-dependent MVB sorting; the additional loss of Ubp7 from these cells restores sorting.[111] Though linked through interaction with Hse1, this finding surprisingly suggested that these two deubiquitinating enzymes perform distinct, and apparently opposing, functions in MVB sorting. Hse1 also associates with the ubiquitin ligase Rsp5, and disrupting the Hse1-Rsp5 interaction leads to MVB missorting in a cargo specific manner.[111] Just as with Bro1, Hse1 appears to link activities of ubiquitination (Rsp5), deubiquitination (Ubp2, Ubp7), and cargo recognition (Vps27-Hse1), with the additional complication that Ubp2 and Ubp7 have distinct impacts on MVB sorting. Together, these observations suggest that the ability of Rsp5 and deubiquitinating enzymes to associate with Hse1 or Bro1 at the endosome is important for sorting a subset of MVB cargoes and supports the model that repeated rounds of ubiquitination and deubiquitination regulate delivery through the endosomal system in yeast. Dynamic ubiquitination of the MVB sorting machinery may also modulate MVB sorting.

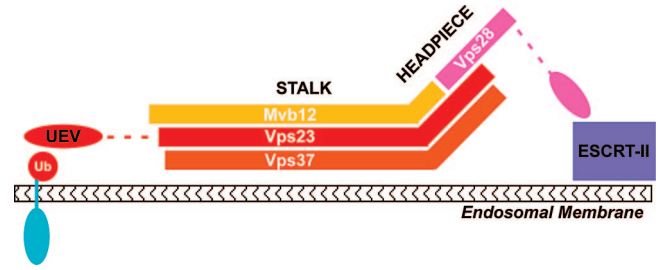

Figure 3. Model for membrane-associated ESCRT-I, ESCRT-II, and cargo. Schematic representation of the ESCRT-I heterotetramer partial structure highlighting the elongated "stalk" region that separates the UEV domain of Vps23 from the "headpiece" region (from which Vps28 contacts ESCRT-II). ESCRT-I interactions with the membrane (via Vps37) and Vps27/Hse1 (via Vps23) have not been highlighted for simplicity. Ubiquitinated cargo is represented by the blue oval with the stalk and red "Ub."

2.3. Machinery Regulating Cargo Selection: Mvb12/ESCRT-I

Our understanding of cargo recognition during MVB sorting has become more complete with the identification of novel ubiquitin-binding motifs within multiple components of the machinery and the alternate recognition mechanisms provided by Rsp5 (as discussed above); the identification of Mvb12 as a new member of yeast ESCRT-I has also refined our understanding of cargo recognition.[114−118] Vps23, Vps28, and Vps37 were initially identified as the ESCRT-I subunits in yeast, and loss of any one of these subunits blocks MVB sorting entirely and gives rise to the altered endosomal morphology known as the Class E compartment.[18,119] Loss of Mvb12 has a different effect on MVB sorting with missorting of some cargoes, including Ste3 and Sna3, while the sorting of other cargoes, including Ste2 and CPS, is less affected.[114,115,117] These phenotypic distinctions indicate that the Vps23, Vps28, and Vps37 complex exhibits residual cargo recognition activity in the absence of Mvb12. Consistent with this concept, the crystal structure of the ESCRT-I core has been determined in both the presence and absence of Mvb12.[24,116,120] ESCRT-I (both with and without Mvb12) exhibits an elongated rodlike structure, which accounts for its high apparent molecular mass when measured by gel filtration chromatography.[119] ESCRT-I has a long "stalk" region consisting of coiled coils contributed by Vps23 and Vps37 as well as Mvb12 (Figure 3). The "head" of ESCRT-I is mainly α-helix hairpins from Vps23, Vps28, and Vps37. Although not crystallized with the core, the Ubiquitin E2 Variant (UEV) domain of Vps23 is presumably positioned off the stalk, facilitating association with ubiquitinated

cargo;[121−124] the carboxyl-terminus of Vps28 (also absent from the ESCRT-I core structures) is similarly presumed to extend from the opposite end of the complex to contact ESCRT-II[24,118] (Figure 3). Mvb12 appears to play an accessory or regulatory role in ESCRT-I function. Consistent with its nonessential role, loss of Mvb12 slightly destabilizes the core ESCRT-I complex[115,117] and alters the ESCRT-I core length;[116] loss of Mvb12 also increases the amount of ESCRT-I that is aberrantly trapped in ILVs formed during MVB sorting.[115] These observations suggest that Mvb12 is important for ESCRT-I recycling and/or the structural stability and shape of ESCRT-I; however, how these functions translate to the cargo-specific defects observed in cells lacking Mvb12 remains unclear. Characterization of additional Mvb12 activities may explain its cargo-specific involvement; however, no functional domains or motifs have been identified within Mvb12 to give hints to its function.

The original "hand-off" model used to describe MVB sorting proposed that Vps27-Hse1 and ESCRT-I bind and concentrate ubiquitinated MVB cargo before transferring the cargo to ESCRT-II. The structure of ESCRT-I raises questions about the model of cargo handoff and/or cooperation between the ESCRTs. The rigid stalk of ESCRT-I would presumably prevent a close interaction of ubiquitinated cargo bound by the Vps23 UEV domain with the ubiquitin binding domains of ESCRT-II (Figure 3). This may indicate that networks of offset ESCRTs are required for the efficient sorting of MVB cargo, or that the hand-off model needs to be refined.

Putative Mvb12 orthologs in mammals (MVB12A and B) and in *C. elegans* (MVB-12) stably interact with their respective ESCRT-I subunits in a 1:1:1:1 ratio. Although they have homology with each other, these proteins have virtually no sequence homology to yeast Mvb12.[125−127] These orthologues are much larger in molecular mass than the yeast Mvb12, but like yeast Mvb12, they do not contain known functional motifs or domains except for the newly described ESCRT-I binding boxes (EBBs) in the carboxyl-terminus of MVB12A and B.[125,126] Both human MVB12A and B interact with the four VPS37 isoforms,[126] suggesting that there may be eight distinct varieties of ESCRT-I in mammalian systems; however, functional distinctions between these complexes have yet to be addressed. Mvb12A and B splice variants lacking the EBBs have also been identified.[126,128] While the truncated MVB12B splice variant does not bind ESCRT-I,[126] the physiological significance of these isoforms is unclear. Disruption of Mvb12-dependent sorting would not be expected to completely block MVB sorting, and depletion of either MVB12A or B does not reduce viral budding. However, MVB12A or B depletion reduces the percentage of mature virions and decreases the infectivity of human immunodeficiency virus (HIV).[126] By contrast, overexpression of MVB12A, MVB12B, or Tsg101 reduced both HIV budding and infectivity. These observations suggest that MVB12A and B have nonredundant regulatory roles in MVB sorting and are consistent with the yeast data that suggest Mvb12 has a role as a regulatory subunit rather than as a core subunit of ESCRT-I. Analysis of the *C. elegans* Mvb12 orthologue, MVB-12, is also consistent with this type of role, as MVB-12 depletion slowed the trafficking kinetics of a model receptor (RME-2, an LDL-like receptor), but not to the same extent observed upon TSG-101 depletion.[125]

The larger Mvb12 orthologues may facilitate the interaction of ESCRT-I with other cellular machinery or permit additional regulation of ESCRT-I. MVB12A can interact with both CD2-associated protein (CD2AP), a scaffold that regulates the actin cytoskeleton, and the structurally related Cbl interacting protein of 85 kDa (CIN85).[128] The E3 ubiquitin ligase Cbl ubiquitinates receptors, including EGFR, to promote their endocytosis and degradation, and CIN85 and MVB12A may coordinate this process with MVB sorting. In addition, the mammalian Mvb12 orthologues are phosphorylated on serine and threonine residues[125,126,128] and tyrosine residues upon EGF stimulation.[128] Mutation of several of these phosphorylation sites to alanine residues in MVB12A prevented the dominant negative effect of MVB12A overexpression on HIV release and infectivity.[126] These observations suggest new methods of Mvb12 regulation and potentially additional aspects of ESCRT-I function.

3. Late-Acting ESCRT Components

3.1. ESCRT-III Assembly

While the predominant view of MVB sorting involves cargo recognition mediated by the early ESCRT complexes (Vps27-Hse1 and ESCRT-I), an alternative system bypassing these components has also been suggested through analysis of Gag budding determinants (reviewed in ref 129). Ubiquitination of Gag and the Gag PTAP motif interacting with ESCRT-I can serve as viral budding determinants, but an alternative pathway involving the YPxL motif, present in both HIV Gag and the equine infectious anemia virus (EIAV) Gag, engaging ESCRT-III via binding to Alix/Bro1 (independent of ESCRT-I or ESCRT-II) has also been implicated.[130,131] This latter pathway suggests a role for ESCRT-III in MVB sorting downstream from ESCRT-I and ESCRT-II and is consistent with analyses in yeast also placing ESCRT-III downstream from ESCRT-I and -II in MVB sorting.[26,27] Cargo engagement with ESCRT-III either via ESCRT-I and -II or via alternative mechanisms, such as Alix/Bro1, thus appears to be the critical step necessary for MVB sorting. This realization has led to further examination of the mechanisms of ESCRT-III function in MVB sorting.

ESCRT-III differs from ESCRT-I and -II in that ESCRT-III appears to assemble on the membrane in the course of MVB sorting with its disassembly also required for the process to complete;[26,30] in contrast, ESCRT-I and -II form stable complexes that appear to be transiently recruited during MVB sorting.[18,27,119] The ESCRT-III proteins are all of similar size (204−240 aa) and charge (generally basic amino-terminus and acidic carboxyl-terminus) and share a conserved five-helix core structure with a more variable carboxyl-terminal tail, which often harbors a sixth helix[132] (Figure 4). In yeast, six proteins (Vps20, Snf7/Vps32, Vps2, Vps24, Did2/Fti1, and Vps60) comprise the ESCRT-III family; in humans, the ESCRT-III family has extended to 11 members with all but one (CHMP7) clearly related to the yeast homologues.[127,130,133] These ESCRT-III subunits can be further divided into the four core subunits (Vps20/CHMP6, Snf7/CHMP4, Vps2/CHMP2, and Vps24/CHMP3), whose functions are critical for MVB sorting, and the two accessory subunits (Did2/CHMP1 and Vps60/CHMP5), which are suggested to have regulatory roles coordinating ESCRT-III assembly and disassembly.[26,29,32,134] The core subunits have been functionally subdivided into the Vps20-Snf7 and the Vps2-Vps24 subcomplexes with Vps20-Snf7 implicated in functioning upstream of Vps2-Vps24.[26] Membrane-associated ESCRT-II (Vps25) interacts directly with ESCRT-III via

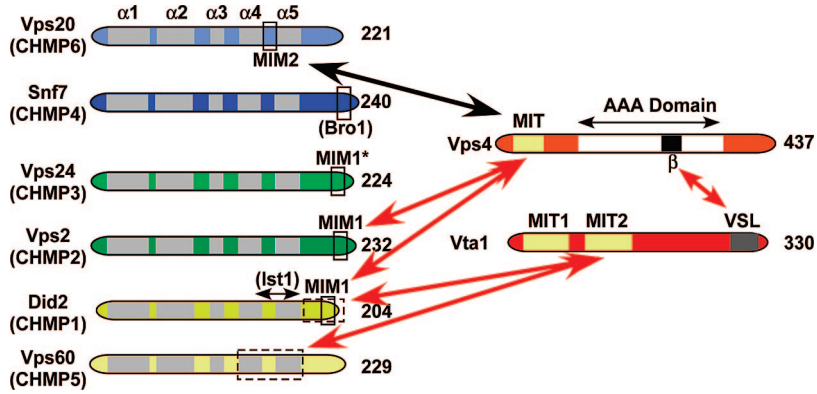

Figure 4. Interactions between Vps4, Vta1, and ESCRT-III. Six ESCRT-III proteins are present in yeast and share a conserved 5-helix core structure. The carboxyl-termini are more divergent and are implicated in coordinating ESCRT-III interactions with additional components of the MVB sorting machinery, including the AAA+ ATPase Vps4 (orange) and the Vps4 activator Vta1 (red) as well as Bro1/Alix and Ist1 (not shown). Vps4 contains an AAA+ domain involved in ATP hydrolysis, an insert within the AAA+ domain (β domain), and an amino-terminal MIT domain. Vta1 contains two MIT-related domains (MIT1, 2) and a carboxyl-terminal VSL region that binds the Vps4 β domain to stimulate Vps4 ATPase activity. The MIM1 domains present in Vps2 and Did2 interact with the Vps4 MIT domain and can stimulate Vps4 ATPase activity (MIM1*: an incomplete or divergent MIM1 is present in Vps24 and may mediate MIT interaction in some contexts). The MIM2 domain in Vps20 can also interact with the Vps4 MIT domain, but this interaction is distinct from the MIT-MIM1 interaction and does not stimulate Vps4 ATPase activity. Did2 also interacts with Ist1 and Vta1, with the Vta1 interaction motif apparently overlapping with MIM1. Vps60 interacts with Vta1 via the α4-α5 region, and interaction of Did2 or Vps60 with the Vta1 MIT2 domain stimulates ATPase activity of the Vta1-Vps4 complex. [Red arrows indicate interactions that stimulate Vta1-Vps4 ATPase activity.]

Vps20,[25,27] leading to its proper endosomal localization and oligomerization with Snf7. Vps2 and Vps24 are dependent on both Vps20 and Snf7 for their association with the upstream MVB sorting machinery,[27] and Did2 is dependent on Vps2 and Vps24 for its recruitment.[134] Vps60 appears to be the final ESCRT-III subunit recruited.[29] These observations have suggested that orchestrated assembly of the ESCRT-III subunits into an oligomeric complex is required for MVB sorting to occur (Figure 5), although how this assembly and subsequent disassembly translate into ILV remains unclear.

Two models have been proposed that may explain the contributions of ESCRT-III to vesicle formation. In the first, ESCRT-III assembly serves to deform the membrane itself and must be released by Vps4 to allow vesicle budding to complete. In the second model, ESCRT-III serves as an adaptor by which force generated through Vps4 ATP hydrolysis leads to vesicle budding. Alternatively, aspects of both of these models may function together to mediate ILV. Consistent with the first of these models, ESCRT-III oligomerization has been suggested to support membrane deformation consistent with the topology of MVB formation. The core ESCRT-III subunits Snf7-1 (CHMP4A) and Snf7-2 (CHMP4B) were overexpressed, and electron microscopy was performed on both "unroofed" cells that were lysed during sample preparation as well as whole cells. Self-assembled circular arrays were found on the plasma membrane and were associated with membrane protrusions, buds, and tubules.[135] These Snf7 filaments were able to interact with an ATPase activity-deficient form of Vps4B, suggesting these filaments are Vps4 substrates. Expression of an ATPase defective form of Vps4B was also able to form a similar phenotype, with large rings on the plasma membrane. The fact that circular arrays were seen at membranes and appeared to promote their negative distortion is the best evidence to date for the model of ESCRT-III-driven ILV formation; however, these experiments do not eliminate the possibility that ESCRT-III is interfacing with effectors to execute membrane deformation. The formation of filamentous ESCRT-III polymers has also recently been observed in vitro utilizing purified Vps24 or mixtures of truncated CHMP2A/

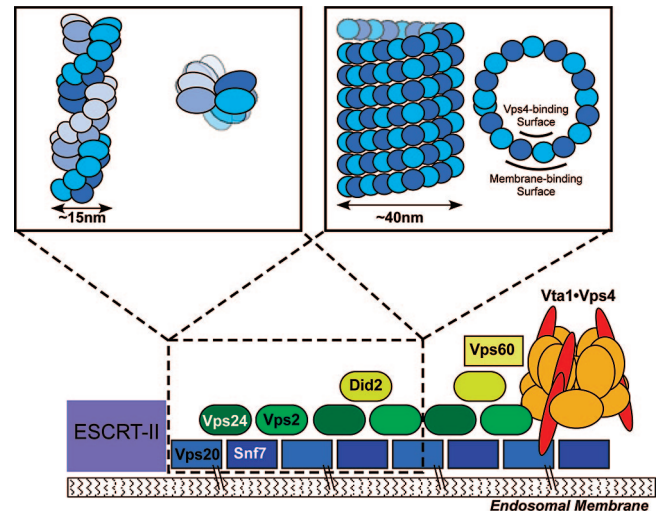

Figure 5. ESCRT-III assembly and disassembly. Yeast ESCRT-III appears to dynamically assemble and disassemble during MVB sorting. Functional analyses have suggested that subcomplexes exist within ESCRT-III. Vps20 and Snf7 appear to be the first subcomplex recruited, in part through ESCRT-II association with Vps20. Vps2 and Vps24 are then recruited in a Vps20- and Snf7-dependent manner. These four subunits comprise the core of ESCRT-III and can execute basal MVB sorting. Efficient MVB sorting also requires the accessory ESCRT-III subunits Did2 and Vps60. Did2 recruitment is dependent on Vps24 and Vps2, while Vps60 appears to be the last subunit recruited. While a layered organization is presented to illustrate this sequential recruitment, ESCRT-III appears instead to polymerize into fibrils to facilitate membrane deformation. Electron microscopy reconstructions of Vps24 homo-oligomers (inset left) or hetero-oligomers of truncated CHMP2/Vps2 and CHMP3/Vps24 (inset right) have identified two distinct assemblies of ESCRT-III that may be pertinent to membrane deformation. Disassembly of ESCRT-III is mediated by the Vta1-Vps4 complex. Interaction of the Vps20 MIM2 with the Vps4 MIT domain as well as association between Vps4 and the Did2-associated factor Ist1 (not shown) have been implicated in facilitating Vta1-Vps4 recruitment. Interactions between Vps4 and MIM1 elements in Vps2 and Did2 as well as between Vta1 and Did2 or Vps60 then potentiate Vta1-Vps4 ATPase activity to stimulate ESCRT-III disassembly.

Vps2 and CHMP3/Vps24;[136,137] these polymers suggest two possible modes of assembly that may have implications for

ESCRT-III membrane deformation in vivo (Figure 5). However, assembly of ESCRT-III apparently is not in itself sufficient for ILV formation as disruption of Vps4 function blocks MVB sorting.[30]

3.2. ESCRT-III Disassembly

Understanding the contribution of Vps4 to ESCRT-III function thus has become a critical issue in MVB sorting, and two general models have emerged suggesting that Vps4 could function as an ESCRT-unfolding machine or as a force generator. Vps4 is a member of the AAA+ ATPase family (ref 138 and reviewed in refs 139 and 140). Other members of this family include the ERAD-chaperone p97, the SNARE-regulator NSF, the bacterial unfoldase ClpX, the microtubule severing protein spastin, and the microtubule motor dynein. Single or dual hexameric rings of AAA+ domains are characteristic of this family, and formation of the ring appears to be required for ATP hydrolysis. While dynein heavy chain harbors six AAA+ domains, proteins with single (type 1) or dual (type 2) AAA+ domains per subunit are more prevalent. Type 2 AAA+ proteins, including p97 and NSF, assemble into hexamers with two rings of AAA+ domains; however, the two rings have been demonstrated to have distinct ATP binding and hydrolysis activities, with one ring that stabilizes the oligomer while the second ring hydrolyzes ATP to remodel substrates (refs 141, 142, and reviewed in refs 143). Type 1 AAA+ proteins have been observed to adopt both hexameric and dodecameric forms, and ATP binding has been suggested to play a critical role in promoting oligomerization. Vps4 has been suggested to form a decamer,[30] dodecamer,[144,145] or tetradecamer,[146] and cryo-electron microscopy reconstruction of Vps4^{E233Q} in the presence of ATP supports the dual hexameric ring assembly model.[144] Vps4 has been suggested to follow a cycle of ATP-stimulated oligomerization, oligomerization-stimulated concerted ATP hydrolysis, and subsequent dissociation back to the monomeric state.[30] ESCRT-III has also been suggested to impact this ATPase cycle. In addition to its AAA+ domain, Vps4 contains an amino-terminal MIT domain that mediates ESCRT-III binding (further discussed below), and ESCRT-III binding has been suggested to promote Vps4 endosomal association and oligomerization.[30,147] According to the model, subsequent concerted ATP hydrolysis by Vps4 dissociates ESCRT-III, suggesting a mechanism by which ESCRT-III assembly and disassembly could be coordinated.

Refinement of this model has been afforded by a series of more recent studies. In particular, the Vps4 cycle necessitating dissociation of the oligomer upon ATP hydrolysis has been called into question. First, Vta1 has been demonstrated to be a positive modulator of Vps4 in vivo, and biochemical characterization has demonstrated that Vta1 stimulates Vps4 ATPase activity.[32,148−150] This stimulation is in part attributable to Vta1 promoting Vps4 oligomerization, and determination of the structure of the Vta1 VSL region that mediates stimulation suggests a possible mechanism.[148,151] The VSL region forms a homodimer with two surfaces on opposite sides that have been implicated in binding the Vps4 β domains, an insert in the AAA+ domain unique to Vps4[149,151,152] (Figure 4); by concurrently contacting β domains extending from the upper and lower rings of the Vps4 oligomer, Vta1 may stabilize the oligomeric form. This mechanism suggests that the Vta1-Vps4 complex may be stable throughout multiple rounds of ATP hydrolysis. The second series of observations contradicting Vps4 dissociation during the ATPase cycle are the cryo-electron microscopy reconstructions. This study indicated that the two rings of the Vps4 dodecamer adopt distinct conformations.[144] This asymmetry is similar to the structures observed with type 2 AAA+ proteins, wherein one ring serves to stabilize oligomerization while the second ring more rapidly hydrolyzes ATP to remodel substrates. This similarity suggests that ATP hydrolysis may occur differentially across the rings of the Vps4 oligomer and may thus stabilize the oligomeric form. Studies of the type 1 AAA+ protein ClpX also support the model of a more stable oligomeric form of Vps4. The active ClpX normally comprises six monomers. However, the subunit composition required for ClpX unfolding activity has been addressed by expressing covalently linked subunits.[153] Even a single active subunit within the AAA+ ring was sufficient to exhibit ClpX ATP hydrolysis and unfolding activities, indicating that concerted ATP hydrolysis throughout the AAA+ ring is not required for ClpX function. This observation suggests that concerted ATP hydrolysis may also not be required within other AAA+ ATPases, including Vps4. However, these observations will need to be reconciled with the result that an ATPase-deficient form of Vps4 acts as a dominant negative in vivo.[138,154] These studies imply that the Vta1-Vps4 oligomer does not dissociate itself during ATP hydrolysis and ESCRT release, but this feature has not been explicitly addressed.

The amino-terminal MIT (microtubule interacting and transport/trafficking) domain of Vps4 plays a critical role coordinating Vps4 interaction with ESCRT-III and stimulation of Vps4 activity. The MIT domain is found in a number of trafficking proteins, including proteins involved in MVB sorting such as the deubiquitinating enzymes AMSH and UBPY.[147,155−157] Structure determination of the Vps4 MIT domain revealed a three-helix bundle reminiscent of an incomplete Tetratricopeptide-like repeat (TPR).[156,158] TPR motifs are protein−protein interaction modules that contain two antiparallel α-helices connected by a small hinge and are often found in multiple copies.[159] Several ESCRT-III core and accessory subunits have been described to bind Vps4 via the MIT domain in both yeast and mammals, including Did2/CHMP1, Vps2/CHMP2, and Vps20/CHMP6[31,33,35,134,156] (Figure 4). For both Did2 and Vps2, a MIT-interaction motif (MIM1) present in the carboxyl-terminus mediates interaction with Vps4.[33,35] However, a distinct motif (MIM2) in the loop between the fourth and fifth α-helices mediates the Vps20-Vps4 interaction.[31] MIT-MIM interactions are required for Vps4 function in vivo, as mutations in Vps4 that disrupt either of these interactions disrupt Vps4 function. However, MIM1 and MIM2 interactions appear to contribute to Vps4 function in distinct manners. Crystal structures of MIT-MIM complexes yielded two surprising results. First, although the MIT domain resembles three helices of the TPR structure, the MIM elements do not complete the TPR 4-helix bundle. The MIM1 helix binds across the MIT rather than in a parallel or antiparallel manner,[33,35] while the MIM2 binds as an extended strand rather than as a helix.[31] The second surprising finding was that the MIT interaction surfaces for MIM1 and MIM2 are distinct. The Vps4 MIT α2-3 surface mediates MIM1 binding for Did2 and Vps2 interactions, while the MIT α1-3 surface binds Vps20 MIM2. These distinct interactions correlate with the differential abilities of Vps2, Did2, and Vps20 to regulate Vps4 ATPase activity: Vps2 and Did2 stimulate Vps4 ATPase activity in a MIT-dependent manner, while the Vps20-Vps4 interaction has not

been demonstrated to enhance ATPase activity.[29] This distinction suggests that Vps4 MIT interactions with MIM1 and MIM2 have discrete consequences with only MIM1 facilitating stimulation of Vps4 ATP hydrolysis.

3.3. Modulation of Vps4

3.3.1. Stimulation by Vta1/Lip5

The Vps4 activator Vta1/Lip5 also harbors 2 MIT-like domains (MIT1 and 2) in its amino-terminus that contribute to stimulation of Vps4.[29,151] Vta1 and Lip5 bind to the ESCRT-III subunits CHMP5/Vps60 and CHMP1/Did2, while Lip5 has been observed to bind CHMP2/Vps2 and CHMP3/Vps24 as well[29,32,34,106,148,160,161] (Figure 4). Did2 and Vps2 harbor MIM1 elements in their carboxyl-termini, and the Vta1-Did2 and Lip5-CHMP1 interactions have been mapped to this region.[29,34] While Vps4 and Vta1/Lip5 can compete for Did2 binding,[34] additional analyses suggest that these binding elements may be discrete.[29] Consistent with this idea, Vps4 MIT domain surface residues implicated in MIM1 binding are not conserved within the Vta1/Lip5 MIT2 binding surface.[151] This Vta1 MIT2 domain has been implicated in binding to both the Did2 carboxyl-terminus and the Vps60 α4-α5 region, and these interactions enhance Vta1 activation of Vps4 ATPase activity.[29,151] The stoichiometry of Vta1-Vps4 binding appears to be 1:2,[144] suggesting that the Vta1-Vps4 oligomer contains 12 Vps4 MIT domains and an additional 12 MIT-like domains contributed by 6 Vta1 molecules. ESCRT-III binding to these MIT domains can enhance Vta1-Vps4 ATPase activity either directly or via Vta1.[29]

3.3.2. Positive and Negative Regulation by Ist1

Did2 can stimulate Vps4 ATPase activity through binding both Vta1 and Vps4 MIT domains, but Did2 may also modulate Vps4 activity through the recruitment of Ist1.[162,163] Ist1 has been implicated as a regulator of MVB sorting, although the mechanisms are unclear. While Ist1 is not required for MVB sorting, loss of Ist1 in conjunction with loss of Vta1 or Vps60 disrupts the process.[162,163] In addition, overexpression of Ist1 can compromise MVB sorting.[162] These observations suggested that Ist1 can function as a negative regulator of MVB sorting, and biochemical characterization supports this model. Ist1 can bind to Vps4, and this interaction inhibits Vps4 oligomerization and ATPase activity.[162] This activity contrasts with the ability of Vta1 to stimulate Vps4 through promoting oligomerization, and coincubation of Ist1, Vta1, and Vps4 results in Ist1 inhibition of both Vta1-Vps4 interaction and Vta1-stimulated Vps4 ATPase activity.[162] However, Ist1 also appears to contribute to Vps4 endosomal localization.[162] These observations suggest that Ist1 may provide both positive and negative regulatory roles toward Vps4 in the MVB sorting pathway. One explanation for the striking complexity of these interactions is to allow for fine-tuning of Vps4 function through protein localization as well as stimulation of ATPase activity. Vps20 and the Did2-Ist1 complex may promote recruitment of Vps4 without stimulating Vps4 ATPase activity.[29,31,162] Vps4 alone has a low level of ATPase activity,[138] but Vps4 can interact with both Vps2 and Did2, which stimulate its activity directly.[29] Vta1 association with Vps4 also stimulates Vps4 activity, and the Did2-Vta1 interaction can enhance this stimulation.[29,32,148] Finally, Vps60, which requires Vta1 for endosomal localization, can also stimulate Vps4 activity through Vta1.[29] These interactions could thus regulate the disassembly of the ESCRT-III subunits from the membrane to permit completion of ILV formation and MVB sorting.

4. Concluding Remarks

The MVB sorting process plays a critical role in facilitating the degradation of membrane proteins within the hydrolytic lumen of the lysosome/vacuole. In the past 10 years, the basic framework by which this process occurs has been elucidated. However, there are still many questions that remain unresolved concerning the mechanisms mediating efficient cargo recognition and ILV formation. Recent studies have helped to expand and refine our understanding of MVB sorting, including the following:

(1) While the major signal for cargo inclusion into the MVB pathway is ubiquitination (recognized by ubiquitin-binding domains within the MVB sorting machinery), additional sorting signals have been uncovered that can reside in the cytoplasmic, transmembrane, or lumenal domains of MVB cargoes.

(2) While the predominant roles of ubiquitin ligases are ubiquitination of MVB cargoes, ubiquitin ligases may also contribute to MVB sorting by physically linking cargo to components of the MVB sorting machinery.

(3) The physical associations of ubiquitin ligases with deubiquitinating enzymes suggest both that these opposing activities are coordinated and that cargo ubiquitination may be more dynamic than previously appreciated.

(4) The identification of an additional, accessory subunit of ESCRT-I (Mvb12) suggests that cargo recognition may be regulated to alter flux into the MVB system.

(5) The assembly of ESCRT-III into polymers appears to be linked to membrane deformations consistent with MVB sorting; however, Vps4 ATPase activity is required to complete this process. The stimulation of Vps4 activity is regulated by ESCRT-III and the ESCRT-III-interacting factors Vta1 and Ist1, suggesting that ESCRT-III assembly and disassembly are coordinated to complete ILV formation and MVB sorting.

5. Acknowledgments

We would like to apologize to those researchers whose related work we were not able to cite in this review. We would also like to thank the members of the Horazdovsky and Katzmann laboratories for stimulating conversations, Ishara Azmi, Bruce Horazdovsky, Zhaohui Xu, and Markus Babst for discussions of Vps4 function, and Andrew Norgan for helpful comments regarding this manuscript. This work was supported by R01 GM73024 to D.J.K.

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