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Role of Ubiquitin and Ubiquitin Ligases in Retroviral Budding

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ROLE OF UBIQUITIN AND UBIQUITIN LIGASES IN RETROVIRAL BUDDING

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The final stage in the production of infectious retroviral particles is the scission of membrane tethers connecting nascent virions to the host cell. Efficient retrovirus release requires late domains, short peptide motifs within the structural Gag proteins that engage the cellular Class E vacuolar protein sorting (VPS) machinery. Three classes of late domains have been described in retroviruses, each with its own cellular interaction partners: PT/SAP domains bind Tsg101, LxxLF or YPXL domains bind ALIX, and PPxY domains bind Nedd4-like HECT ligases, a class of ubiquitin ligase enzymes. The functional link between HECT ligases and the VPS pathway has not been defined, but multiple lines of evidence suggest that ubiquitin is generally important for late domain activity. Retroviral Gag proteins are often monoubiquitinated, and depletion of free cellular ubiquitin results in accumulation of assembled but immature virions at the cell surface, attached to the cell by membranous stalks. Moreover, mutation of multiple ubiquitin acceptor sites (lysine residues) in HIV-1 and RSV Gag proteins markedly inhibits budding. However, the precise role of ubiquitin in retroviral particle release is poorly understood.

To test the importance of direct Gag ubiquitination for viral particle release we constructed a prototype foamy virus (PFV)-derived Gag protein devoid of lysine residues, which can assemble at the plasma membrane in the absence of other viral protein expression. Efficient release of lysine-free PFV Gag virus-like particles (VLPs) required

the PSAP late domain and an intact Class E VPS pathway. Remarkably, the MLV PPXY late domain could functionally replace the PSAP motif in lysine-free Gag, retaining its capacity to engage HECT ubiqutin ligases and support particle release. Moreover, overexpression of catalytically active HECT ligases markedly stimulated PPxY-dependent lysine-free PFV Gag budding, while catalytically inactive mutants did not. Interestingly, the ability of different HECT domains to promote PFV VLP release correlated with their autoubiquitination activity, but not with their ability to ubiquitinate a Gag substrate containing late domain-proximal lysine residues.

Importantly, direct fusion of ubiquitin to the C-terminus of lysine free PFV Gag rescued budding in the absence of conventional late domains. Additionally, ubiquitin fusion dramatically enhanced PSAP-dependent budding. This effect was observable only when a large fraction of Gag carried ubiquitin. Ubiquitin-dependent particle production was abolished by overexpression of dominant negative Vps4 and disruption of the Ile44 hydrophobic patch in ubiquitin. Individual depletion of Tsg101 and ALIX partially suppressed ubiquitin-dependent budding, and the inhibition was enhanced by simultaneous depletion of the two factors. Thus, multiple ubiquitin-binding Class E factors may provide redundant mechanisms for engaging the VPS machinery, which is essential for ubiquitin-dependent budding.

Our findings suggest that deposition of ubiquitin at sites of virion assembly by HECT ligases provides docking sites for class E factors, in effect serving as an alternative or additional late domain. However, since both lysine free and ubiquitin fused versions of PFV Gag proteins support budding, the precise nature of the ubiquitin acceptor, be it the Gag protein itself or trans-acting factors, appears unimportant.

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Chapter I. Introduction

Retroviruses comprise a diverse family of RNA viruses that infect virtually all vertebrates. They are notable for their ability to establish persistent infection by making DNA copies of their genomes and integrating them into the host's genomic DNA [1], using virally encoded reverse transcriptase [2] and integrase enzymes [3-5]. Though most retroviruses are relatively benign, several members are highly pathogenic and have been associated with tumorigenesis [6-8] and immune deficiency [9]. An enormous amount of research in recent decades has been devoted to the study of human immunodeficiency virus (HIV)-1, the causative agent of AIDS, which has killed over 25 million since its discovery. However, retroviruses also hold great interest as vectors for delivery of gene therapy and markers of evolutionary history.

Retroviral particles consist of a membrane-enveloped protein shell that collapses upon maturation to form a dense spherical, conical, or cylindrical inner core (reviewed in [10,11]). This core, or capsid, carries essential viral enzymes and two single-stranded linear copies of the RNA genome, tightly complexed with nucleocapsid proteins. The phospholipid bilayer that covers the virion is derived from the host cell membrane and contains highly glycosylated envelope proteins, which protrude from the surface and determine host cell tropism.

Retrovirus replication cycle

The infectious cycle (illustrated in Fig. 1) begins with the initial attachment of virions to target cells by interaction with various molecules expressed on the cell surface

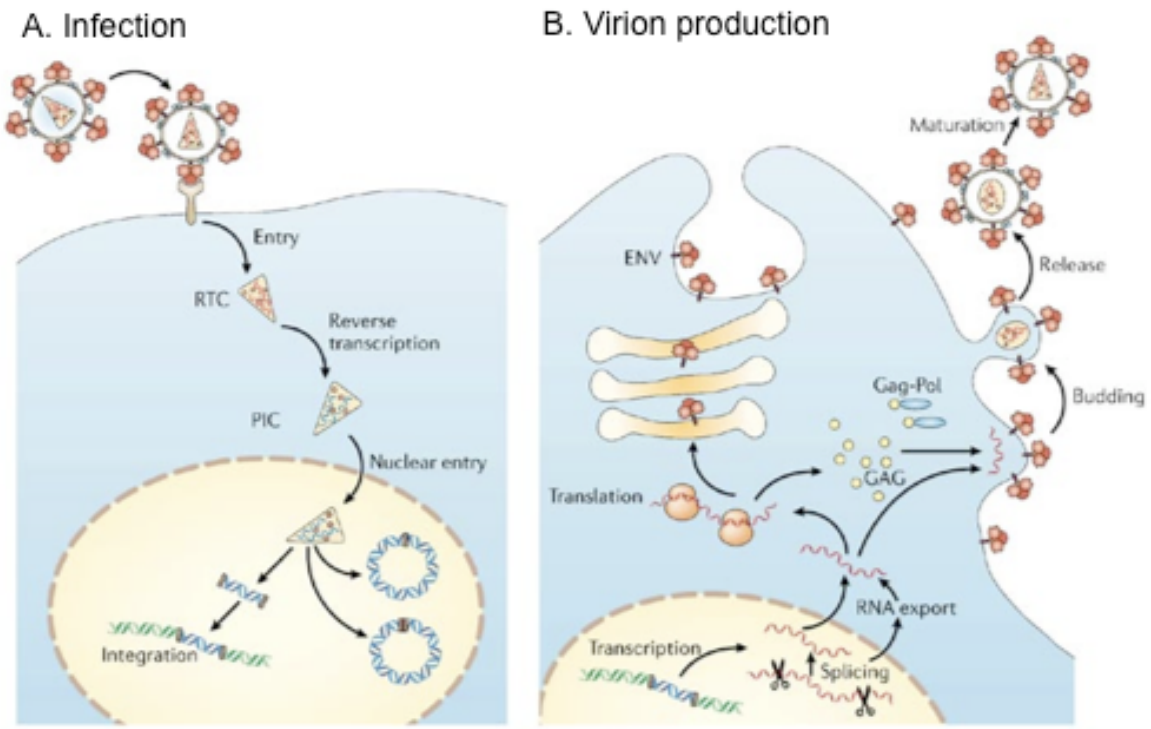


Figure 1. The retroviral lifecycle. (A) Typical route of cell infection and integration into host genome. (B) Typical route of virion production, including gene expression, assembly, and release. Taken from Goff, Nature Reviews Microbiology, 2007 [12].

[13,14]. This concentration of viral particles at the plasma membrane facilitates the recognition and binding of specific host receptors by the surface-binding region of viral envelope proteins [15-18]. A series of conformational changes within the transmembrane region of envelope proteins mediates the fusion of viral and cellular membranes, allowing the release of the capsid into the cytoplasm (reviewed in [19,20]). Fusion may be triggered directly by receptor binding at the plasma membrane [21] or by exposure to low pH in acidified endosomes following internalization [22-24]. Although some DNA synthesis may begin in the virion [25-27], the majority of reverse transcription in most retroviruses occurs soon after entry into the host cell and may be triggered by the high concentration of deoxyribonucleotides in the cytoplasm [28,29]. Upon entry the viral core

begins a process of progressive disassembly, known as uncoating [29-31]. The pre-integration complexes (PICs), consisting of viral cDNA and associated enzymes, structural and accessory proteins, migrate toward the nucleus by engaging the cellular actin cytoskeleton and/or microtubule network and associated motor proteins [30,32-34].

In order to access the host cell genome, retroviral PICs must penetrate the nuclear membrane. Since lentiviruses can infect non-dividing cells [35-38], their PICs presumably enter the nucleus through the nuclear pore. Several lentiviral proteins and the viral DNA itself have been reported to possess nuclear targeting elements [39-44]; however, none of them have been unequivocally shown to be absolutely required for PIC nuclear import [45-48]. Most retroviruses must wait for nuclear envelope breakdown during mitosis to access the nucleus and thus depend on the cell cycle for productive infection [37,49,50]. The viral integrase enzyme catalyzes the double transesterification reaction to insert the viral cDNA into the host genome [51-54]. Although integration site selection is generally not sequence-specific, it may be influenced by regional chromatin structure [55,56] and interactions between integrase and chromatin-binding proteins [57-60]. Retroviral integration is essentially permanent and allows the viral genome to persist as a provirus for the lifetime of the cell.

The cellular transcription machinery treats the provirus as a host gene, generating a capped and polyadenylated viral mRNA (reviewed in [61]). Most proviral promoters, located within the long terminal repeat (LTR) regions, are constitutively active; however, some LTR promoters require transactivation by virally encoded regulatory factors [62]. Nuclear export of unspiced or partially spliced viral mRNA requires interaction of specific RNA structural elements with the host RNA export machinery, either directly or

through the activity of viral cofactors [63-66]. Simple retroviruses encode only the *gag*, *pol*, and *env* genes, while complex viruses encode additional regulatory or accessory genes. The *gag* gene codes for the structural proteins that drive particle assembly and make up the viral core [67,68], *pol* codes for essential enzymatic functions [2-4,69], and *env* codes for the envelope glycoproteins that mediate host receptor recognition and membrane fusion. The Gag structural precursor is translated by free ribosomes from unspliced RNA. In most retroviruses, one in every 10-20 translation events results in readthrough [70,71] or frameshifting [72,73] at a slippery sequence before the Gag stop codon, generating the Gag-Pol polyprotein. Env proteins are translated from singly spliced subgenomic mRNAs on membrane-bound ribosomes in the endoplasmic reticulum, where they fold, oligomerize, and become heavily glycosylated. En route to the plasma membrane, Env traffics through the Golgi, where it gets cleaved by furin-like proteases to generate surface and transmembrane subunits (reviewed in [12]).

In most retroviruses assembly of extracellular virions is driven primarily by Gag, and expression of the Gag protein in the host cell is sufficient for the formation and release of virus-like particles (VLPs) (Fig. 2). The M (membrane binding)-domain of Gag mediates membrane association through interactions between a patch of hydrophobic and basic residues near the amino terminus with membrane phospholipids. Fatty acid (myristate) modifications of some retroviral Gag proteins facilitate membrane targeting by inserting into the plasma membrane [74-77]. The myristate may be buried in a hydrophobic pocket of Gag and exposed upon Gag polymerization [78-81]. The I (interaction)-domain within the C-terminal half of Gag mediates Gag-Gag interactions that drive particle assembly [82-84]. The L (late budding)-domain, which may be located

either within the N-terminal third or at the C-terminus of Gag, is essential for efficient membrane separation between the fully assembled virion and the host cell (reviewed in [85]). Two major routes of assembly have been observed for different viruses. In some viruses Gag is targeted directly to the plasma membrane [86,87], where lateral Gag-Gag interactions and bud growth induce outward membrane curvature. Other viruses pre-assemble in the cytoplasm before trafficking to the plasma membrane for envelopment and release [88,89].

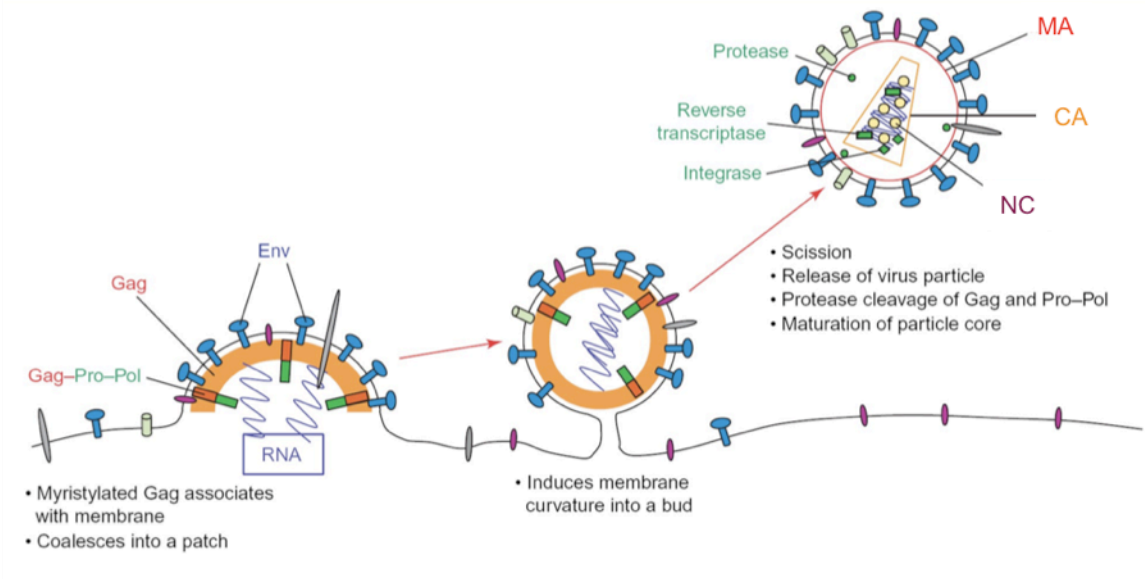


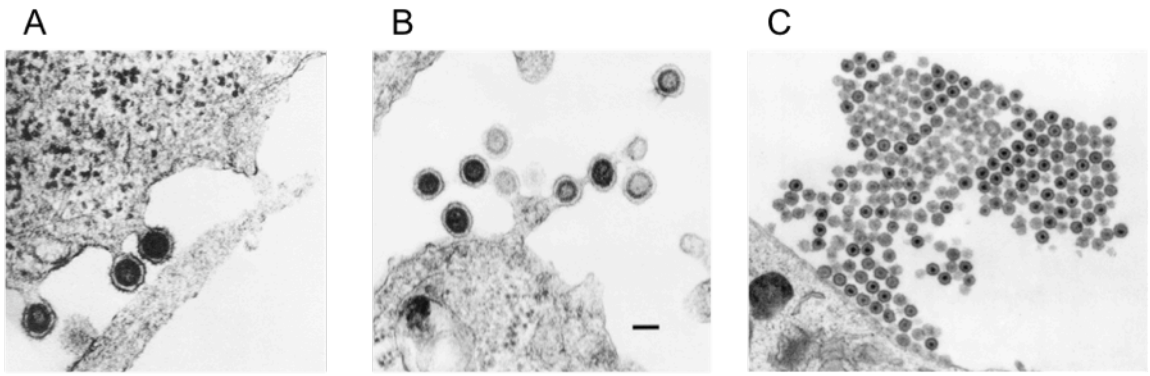
Figure 2. Assembly of HIV-1 particles. Gag proteins associate with cellular membranes, and their oligomerization induces membrane curvature. Viral enzymes are typically incorporated into virions as part of Gag-Pro-Pol polyproteins, which co-assemble with Gag. Other viral components are incorporated through interactions with Gag. After particle release, the viral protease cleaves Gag into MA, CA, and NC and releases the viral enzymes to generate mature infectious virions. Adapted from Pelchen-Matthews, et. al. *Trends in Microbiology*, 2004 [90].

Pol typically gets incorporated into virions as a Gag-Pol polyprotein, which associates with membranes as it co-assembles with Gag, thereby allowing the virus

control over the stoichiometry of structural and enzymatic components in the virion. Env is thought to be incorporated through interactions between its cytoplasmic tail and the N-terminus of Gag [91]. Env may also target Gag to specific membrane domains [92-94]. The viral RNA contains packaging signals near the 5' end, which mediate its incorporation into nascent virions by interactions with basic Cis-His sequences in the nucleocapsid region of Gag [95,96]. The RNA is packaged as a dimer, held together by base-pairing at specific palindromic sequences, known as dimer linkage structures (DLS) [97,98].

Formation of mature infections particles requires cleavage of the Gag and Gag-Pol precursors by the viral protease to generate separate matrix (MA), capsid (CA), and nucleocapsid (NC) structural proteins, and protease (PR), reverse transcriptase (RT), and integrase (IN) enzymes [69]. NC forms a tight complex with the viral RNA inside the CA core, while MA remains closely associated with the inner leaflet of the lipid envelope. Maturation is closely linked to budding, and protease activation may be induced by its oligomerization during Gag and Gag-Pol co-assembly. However, additional triggers must occur, since viruses that assemble in the cytoplasm mature only after their release from the host cell [99].

The membrane fission event that separates nascent virions from the host cell, and from each other, is not spontaneous. Retroviral Gag proteins and structural proteins of some other enveloped viruses encode short peptide motifs, called late (L)-domains, which are essential for efficient virus particle release [100-102]. Inhibition of late domain function causes fully assembled immature virions to remain tethered to the cell surface by a continuous membranous stalk (Fig. 3A, B, and C) [100]. Three types of late domains



D

Viral late budding domains (L domains):

HIV-1	PEPTAPPEE……YPLASLRSLFG
MLV	LLTEDPPPYRD
RSV	TASAPPPPYVG
EIAV	TPQTQNLYPDLSEIK
HTLV-1	DPQIPPPPYVEPTAPQV
MPMV	PPPYNKATPSAPTVM
Ebola	MRRVILPTAPPEYMEAI
VSV	LGIAPPPYEEDTSMEYAPSAP

Figure 3. Late domains promote efficient separation of fully assembled virions from the host cell. (A-C) Thin section electron microscopy analysis of (A) MLV with a mutated PPPY motif [103], (B) MLV in cells overexpressing dominant negative Vps4 E228Q protein [103], (C) RSV in cells treated with proteasome inhibitor MG-132 [104]. (D) Examples of late domains from retroviruses, filoviruses (Ebola), and rhabdoviruses (VSV).

have been described in retroviruses, containing the PT/SAP [100,101], PPxY [105,106], or YPxL/LxxLF [107,108] sequence motifs (Fig. 3D). Late budding defects caused by mutation of these motifs can be rescued by introduction of heterologous late domains. Moreover, late domains have been shown to retain their activity when transferred to different positions within Gag, suggesting that they function as docking sites for cellular factors rather than as essential structural elements [109,110]. Interestingly, several enveloped viruses encode multiple different late domain motifs in close proximity, with one usually playing a dominant role in promoting particle release [108,111,112]. Many aspects of late domain function have been elucidated over the past decade. The cellular binding partners of retroviral late domains have been described: PT/SAP domains recruit Tsg101 [103,113,114], LxxLF or YPxL domains recruit AIP-1/ALIX [108], and PPxY domains recruit Nedd4-like HECT ubiquitin E3 ligases [111,115-121]. All of these late domains require an intact cellular Class E vacuolar protein sorting (VPS) pathway to promote virion release [103,108,115,122-124].

Cellular vacuolar protein sorting machinery

The VPS pathway mediates the formation of intralumenal vesicles (ILVs) in multivesicular bodies (MVBs) (Fig. 4B), a process that involves membrane evagination away from the cytoplasm and is therefore topologically equivalent to viral budding. The pathway plays a critical role in the downregulation of activated cell surface receptors [125,126]. Endocytosed ligand-bound receptors can be either recycled to the plasma membrane or delivered to the lysosome (vacuole in yeast) for degradation. As late

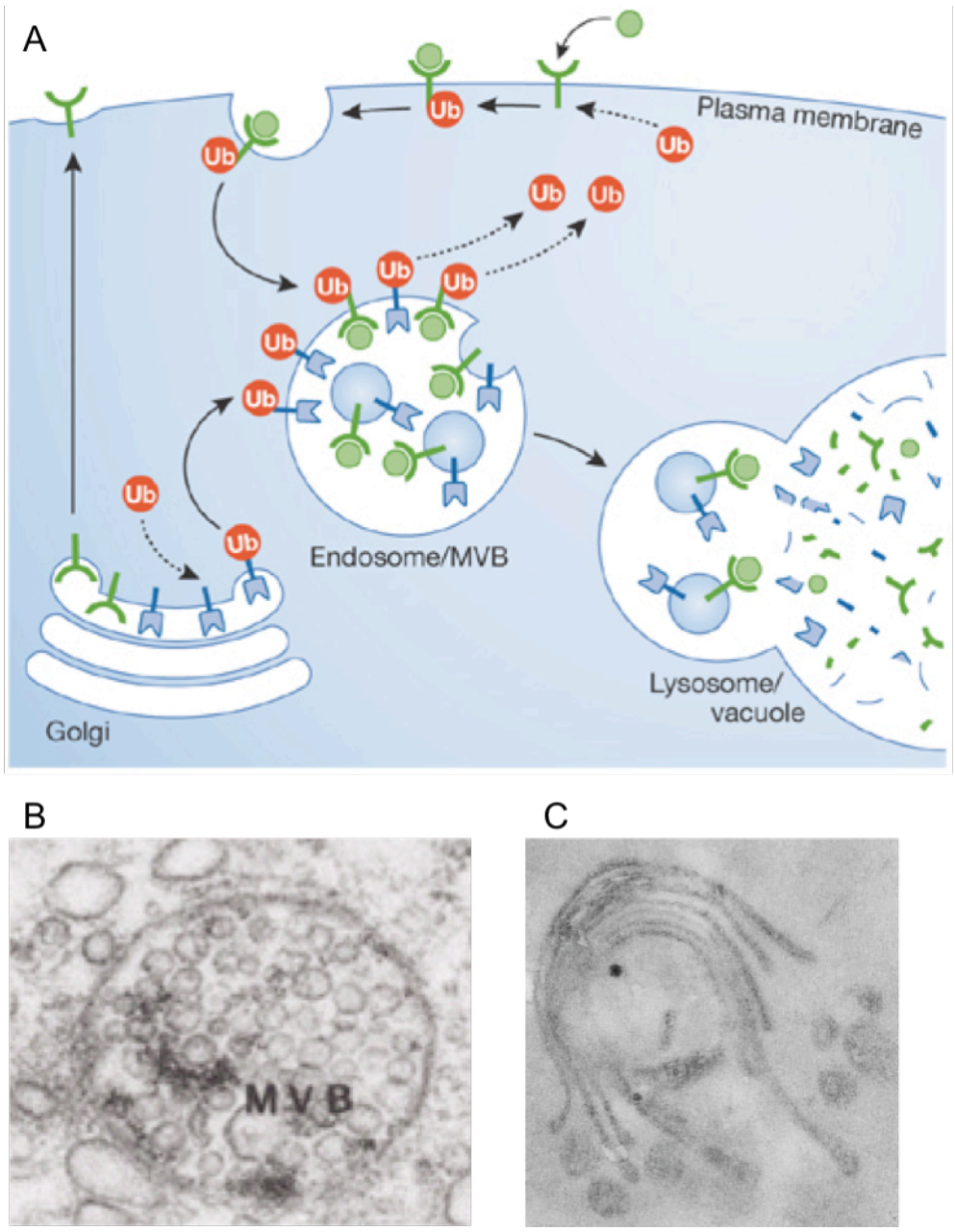


Figure 4. Vacuolar protein sorting pathway. (A) Ubiquitinated cell surface receptors are internalized and delivered to endosomes, which also receive cargoes from the trans-Golgi network. As endosomes mature, vesicles bud into the lumen to form multivesicular bodies (MVBs). Some cargoes are recycled back to the plasma membrane, either directly or indirectly through recycling endosomes. Other cargos are sorted onto intraluminal vesicles, which eventually get degraded by proteolytic enzymes when multivesicular bodies fuse with lysosomes. Taken from Nickerson, et. al. *EMBO Reports*, 2007 [127]. (B) Transmission electron micrograph of a multivesicular body [128]. (C) Aberrant "Class E" compartment caused by Vps4 deletion in yeast [125].

endosomes mature, soluble VPS factors are recruited to the limiting membrane, where they promote the formation and release of vesicles into the endosome lumen and regulate the sorting of proteins onto ILV membranes. Fusion of mature MVBs with the lysosome exposes the ILVs and their associated proteins to hydrolytic enzymes, resulting in their degradation. Proteins on the limiting membrane of MVBs remain intact and may subsequently get recycled to the cell surface (Fig. 4A). Disruption of the VPS machinery in yeast results in the formation of abnormally large, multilamellar 'Class E' compartments that lack intralumenal vesicles (Fig. 4C) and in the mislocalization of endocytic cargo proteins to the vacuolar membrane [125].

Ubiquitin modification of endocytic cargo is a key signal for sorting onto intralumenal vesicles [126]. Ubiquitin is a small (76 residue) polypeptide, highly conserved among eukaryotes, that becomes covalently attached to other proteins by an isopeptide bond between the exposed carboxyl group of its C-terminal glycine residue and the ε-amino group of lysine residues in the substrate protein (reviewed in [129,130]). Since ubiquitin contains 7 lysine residues (Lys6, Lys11, Lys27, Lys29, Lys33, Lys44, and Lys63), it can itself serve as a substrate for ubiquitination, thus forming linear or branched ubiquitin chains [131]. Proteins can be mono- or polyubiquitinated on single or multiple lysine residues. Few instances of ubiquitin modification on non-lysine residues have also been reported [132,133]. Different types of ubiquitin modifications are selectively recognized by various cellular pathways and thus signal different fates for the substrate protein. Among the numerous functions of ubiquitin, the targeting of misfolded or damaged proteins for degradation by the 26S proteasome by K48-linked polyubiquitin chains is the best understood [134]. Lys11-linked ubiquitin chains have recently been

shown to direct endoplasmic reticulum-associated degradation (ERAD) [135]. Monoubiquitination of membrane receptors appears to be sufficient to signal entry into the MVB pathway; however, many endosomal cargos are modified by K63-linked or other types of polyubiquitin chains [126,136-138].

The Class E VPS machinery is highly conserved from yeast to humans, and genetic studies in yeast have been instrumental to the identification of many mammalian VPS factors and their interactions. The human vacuolar protein sorting (VPS) machinery consists of about 30 factors that form several multiprotein complexes, known as endosomal sorting complexes required for transport (ESCRTs) (outlined in Table 1 and Fig. 5, reviewed in [139]). The traditional model for ubiquitinated protein sorting proposes that ESCRT-I, -II, and -III are recruited sequentially to the limiting membrane of MVBs by their interactions with each other and ubiquitinated cargo. ESCRT-III is thought to drive the membrane deformation and scission event required for vesicle budding into the lumen of MVBs [140,141]. The AAA ATPase Vps4 is required for ESCRT disassembly and VPS factor recycling after each round of vesicle formation and release [142,143].

ESCRT-I is thought to be responsible for recognition of ubiquitinated cargo and recruitment of downstream VPS components to endosomal compartments [126]. The major soluble form of the complex is a heterotetramer of Tsg101 (Vps23 in yeast), Vps28, Vps37, and MVB12, containing a single copy of each subunit [144,145]. ESCRT-I is largely cytosolic, but relocalizes to aberrant endosmoal compartments induced by the loss of Vps4 activity, either by deletion of the Vps4 gene (in yeast) [126] or

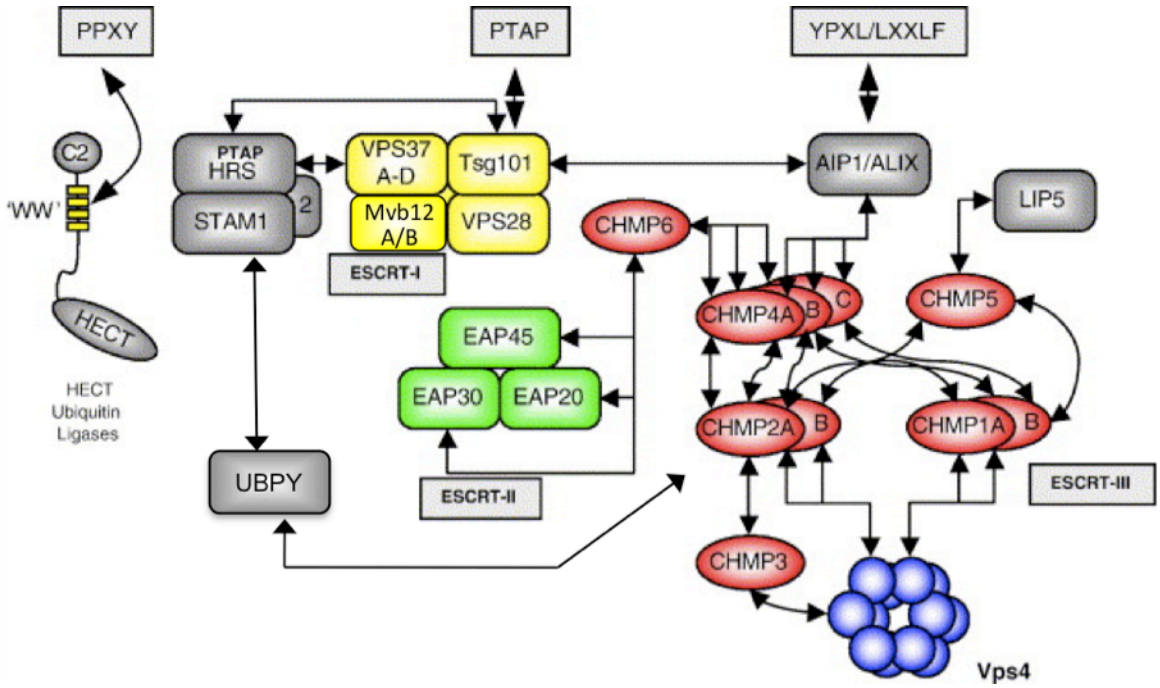


Figure 5. Late domains recruit HECT ubiquitin ligases or components of the Class E vacuolar protein sorting (VPS) pathway. Direct protein-protein interactions are indicated by double ended arrows, and ESCRT-I, -II, and -III complexes are highlighted in yellow, green, and red, respectively. Adapted from Bieniasz, *Virology*, 2006 [85]

overexpression of the catalytically inactive Vps4 mutant protein (in mammalian cells) [146]. Tsg101 can bind ubiquitinated MVB cargo proteins through its ubiquitin E2 variant (UEV) domain, which structurally resembles E2 ubiquitin conjugating enzymes but lacks a conserved cysteine residue essential for catalytic activity [147,148]. Tsg101 also forms a ternary complex with Vps28 and Vps37 via its C-terminal domain [149]. Vps28, in turn, interacts with ESCRT-II through its C-terminal domain and may thus mediate its recruitment to membrane domains enriched with MVB cargo [150]. Expression of Tsg101, Vps28, and Vps37 is essential for MVB biogenesis and cargo sorting [126,146,149]. The recently identified Mvb12 protein interacts with the Tsg101-Vps37 binary complex [145] and modulates ESCRT-I function [145,151-153]. The C-terminus of yeast Mvb12 appears to contain a ubiquitin-binding domain [154]. Though

Mvb12 is not strictly required for MVB sorting, it appears to prevent premature binding of ESCRT-I to ESCRT-II and facilitate its dissociation from cargo, thus preventing its encapsulation within vesicles that bud into the MVB lumen [152]. Mvb12 may also function in cargo selection, as it appears to enhance ubiquitin-independent cargo sorting into the yeast vacuole [153].

Table 1. Class E vacuolar protein sorting factors in yeast and mammals

Complex	Yeast protein	Mammalian protein
ESCRT-0	Vps27	Hrs
	Hse1	STAM1/2
ESCRT-I	Vps23	Tsg101
	Vps28	Vps28
	Vps37	Vps37A/B/C/D
	Mvb12	Mvb12A/B
ESCRT-II	Vps22	Eap30
	Vps25	Eap20
	Vps36	Eap45
ESCRT-III	Vps2	CHMP2A/B
	Vps20	CHMP6
	Vps24	CHMP3
	Snf7 (Vps32)	CHMP4A/B/C
	Did2 (Vps46)	CHMP1A/B
	Vps60	CHMP5
Associated proteins	Vps4	Vps4A/B
	Bro1	Alix

Efficient recruitment of ESCRT-I to endosomal compartments appears to require another complex, sometimes referred to as ESCRT-0, which includes Hrs (Vps27) and STAM (Hse1) proteins [155,156]. Hrs has been shown to be important for ligand-dependent lysosomal degradation of platelet-derived growth factor (PDGF) receptor and epidermal growth factor receptor (EGFR), and its depletion from cells induces the formation of enlarged endosomes [155]. Hrs contains a FYVE domain, which preferentially binds PI(3)P-containing membranes, and a coiled-coil domain, which mediates its association with STAM and contributes to membrane binding [155,157]. Moreover, the central region of Hrs contains a PSAP motif, which is essential for its interaction with Tsg101 [157-159]. Hrs thus recruits STAM and ESCRT-I to early endosomal membranes [155,157,158]. The Hrs-STAM complex can directly associate with ubiquitin via three ubiquitin binding sites in Hrs (one double-sided UIM and one VHS motif) and two in STAM (one UIM and one VHS) [155,156,160,161]. Mammalian Hrs also interacts with Eps15, another UIM-containing protein [155]. Mono- and poly-ubiquitination of membrane proteins serves as a key determinant for their targeting to the lysosome lumen during the critical sorting step in the MVB. Despite the low affinity of individual UIMs and VHS motifs for monoubiquitin, multivalent ubiquitin binding by ESCRT-0 allows it to interact with ubiquitinated cargo with strong avidity [161]. Indeed, ubiquitinated proteins have been shown to co-localize with Hrs and STAM on endosomal membrane microdomains [155]. Thus, ESCRT-0 appears to concentrate ubiquitinated cargo on endosomes while recruiting downstream ESCRT components that mediate endosome maturation by inducing the formation of intralumenal vesicles.

ESCRT-II is a stable soluble complex that functions downstream of ESCRT-I [162]. The complex is a trilobal tetramer, consisting of two copies of EAP20 (Vps25) bound to single copies of Eap30 (Vps22) and EAP45 (Vps36) [163,164]. Deletion of any ESCRT-II component in yeast induces the formation of an aberrant Class E compartment that accumulates endosomal cargo [162]; however, ESCRT-II does not appear to be universally required cargo sorting in mammals [165]. EAP45 can efficiently bind phosphoinositide-3-phosphates and can, therefore, independently mediate ESCRT-II recruitment to endosomal membranes [150,162,166]. Mammalian EAP45 can bind ubiquitin via its N-terminal GLUE domain [166], and both EAP30 and EAP45 have the capacity to bind the C-terminal "steadiness box" of Tsg101 [167]. In contrast, the yeast homolog of EAP45, Vps36, simultaneously mediates ESCRT-II association with membranes, ubiquitin, and ESCRT-I. The GLUE domain of Vps36 contains two NZF zinc fingers, one of which binds ubiquitin and the other Vps28 [150]. EAP20 mediates ESCRT-III recruitment by interacting with the basic N-terminal domain of CHMP6 (Vps20) [150,162,167,168].

ESCRT-III comprises the core machinery of MVB biogenesis that executes the membrane deformation and scission events required for intralumenal vesicle formation [140,141,169]. ESCRT-III proteins exist as soluble monomers in the cytosol, held together in a "closed" autoinhibited conformation by association of their basic amino terminal portion with the highly acidic carboxy terminal portion [124,170,171]. CHMP6 contains a myristoylation signal that allows it to independently bind endosomal membranes [170]. Interaction of EAP20 with CHMP6 on endosomes induces a conformational change in CHMP6, thus activating it to nucleate CHMP4 (Snf7)

oligomerization [169,171,172]. ESCRT-II, therefore, appears to function as a scaffold for ESCRT-III assembly. Overexpressed CHMP4 can polymerize in circular membrane-associated filamentous arrays that deform the membrane away from the cytoplasm [140]. Interestingly, ESCRT-II containing a single EAP20 arm can promote CHMP4 oligomerization but cannot support MVB sorting, suggesting that membrane evagination may require lateral interactions between multiple ESCRT-III filaments [172]. CHMP3 (Vps24) terminates CHMP4 oligomerization and recruits CHMP2 (Vps2), which in turn recruits Vps4 by interactions with its MIT domain [171]. Incubation of spherical liposomes with all four yeast ESCRT-III components in vitro induces the formation of inward invaginations, or buds, which are reversed by addition of Vps4 and ATP. Vps2 (CHMP2) is required for complete detachment of these buds from the limiting membrane, while Vps4 and ATP are required for subsequent rounds of intralumenal vesicle formation [141]. Thus, ESCRT-III mediates membrane scission, and Vps4 allows recycling of ESCRT components.

The mammalian ESCRT pathway appears to have a redundant or alternative mechanism for ESCRT-III recruitment. AIP1/ALIX binds CHMP4 proteins and contains a PTAP motif, which mediates its interaction with the Tsg101 UEV domain [108,122,123]. ALIX thus effectively bridges ESCRT-I with ESCRT-III. Bro1, the yeast homolog of ALIX, interacts directly with Snf7 but lacks a PTAP-like motif and, therefore cannot mediate the recruitment of ESCRT-III to ESCRT-I. Instead, it appears to function as an adaptor for the deubiquitinating enzyme Doa4, whose recruitment to budding vesicles allows the recycling of ubiquitin from cargo proteins before they become sequestered within ILVs [173,174]. Similarly, mammalian ESCRT-III components

recruit deubiquitiniating enzymes AMSH and UBPY to endosomal compartments, where their catalytic activity is stimulated by interaction with STAM [175].

The Vps4 AAA-type ATPase mediates the dissociation of ESCRT components from membranes. Recruitment of ATP-bound Vps4 dimers to endosomal membranes is thought to result in the formation of higher order Vps4 oligomers (hexameric rings) in association with ESCRT complexes via coiled-coil interactions [176]. Oligomerization then triggers the ATPase activity of Vps4, inducing a comformational change that destabilizes the complexes, resulting in ESCRT and Vps4 disassembly and dissociation from the membrane [142]. The catalytic activity of Vps4 may also be modulated by interaction with ESCRT-III and other cellular proteins, including LIP5 and Ist1 [177-182]. One model for ESCRT disassembly suggests that ESCRT-III proteins associate with the central pore of the Vps4 ring in the bud neck of nascent vesicles and are shunted through the pore into the cell interior by conformational changes induced by the ATP hydrolysis cycle [176,182-184]. The loss of Vps4 catalytic activity causes VPS proteins to become trapped on endosomal membranes, resulting in profound defects in MVB biogenesis and cargo sorting [125,142].

The ability of ESCRT-III to induce membrane deformation has been challenged by a recent study, in which yeast MVB biogenesis was reconstituted in vitro using giant unilamellar vesicles (GUVs) [185]. Co-assembly of grossly overexpressed ESCRT-III proteins underneath a membrane into an interior coat can induce membrane curvature and bud formation [140,141,169]. However, intralumenal vesicles formed by such a mechanism would, in principle, consume ESCRT components [141] and deliver them to the lysosome lumen for degradation. At physiological concentrations, ESCRT-III cannot

induce ILV formation on its own. Instead, ESCRT-I and ESCRT-II together deform GUV membranes to produce open buds in the absence of cargo or other ESCRT components. The two complexes localize to the bud neck, and therefore appear to stabilize it, without entering the bud themselves (Fig. 6B [185]). ESCRT-0 seems to possess an intrinsic capacity to cluster and concentrate ubiquitinated cargo on membrane patches (Fig. 6A [155,185]). ESCRT-I recruits ESCRT-0 to the bud neck [157-159], presumably allowing it to deposit ubiquitin-bearing cargo into the forming intralumenal vesicles (Fig. 6B [185]). Meanwhile, ESCRT-II recruits ESCRT-III proteins to the bud neck [185], nucleating the assembly of Snf7 into an interior collar (Fig. 6C [172]) and allowing membrane scission by Vps2 to release the nascent vesicle into the GUV lumen (Fig. 6D [141]). While ubiquitinated cargo localizes to intralumenal vesicles, ESCRT components remain at the bud neck [185], where they can be recycled by Vps4 ATPase activity [141,169].

Another recent study has challenged the model of sequential handover of ubiquitinated cargo from ESCRT-0 to ESCRT-I, -II, and -III [154]. Disruption of ubiquitin binding by either ESCRT-I or ESCRT-II was insufficient to inhibit cargo sorting to the vacuole in yeast. Simultaneous disruption of ubiquitin binding by both complexes impaired sorting of some cargos. Complete inhibition of MVB sorting required disruption of ESCRT-I and ESCRT-II ubiquitin binding and ablation of ESCRT-I interaction with ESCRT-0, suggesting that the complexes provide parallel mechanisms for funneling ubiqitinated cargo into MVB pathway.

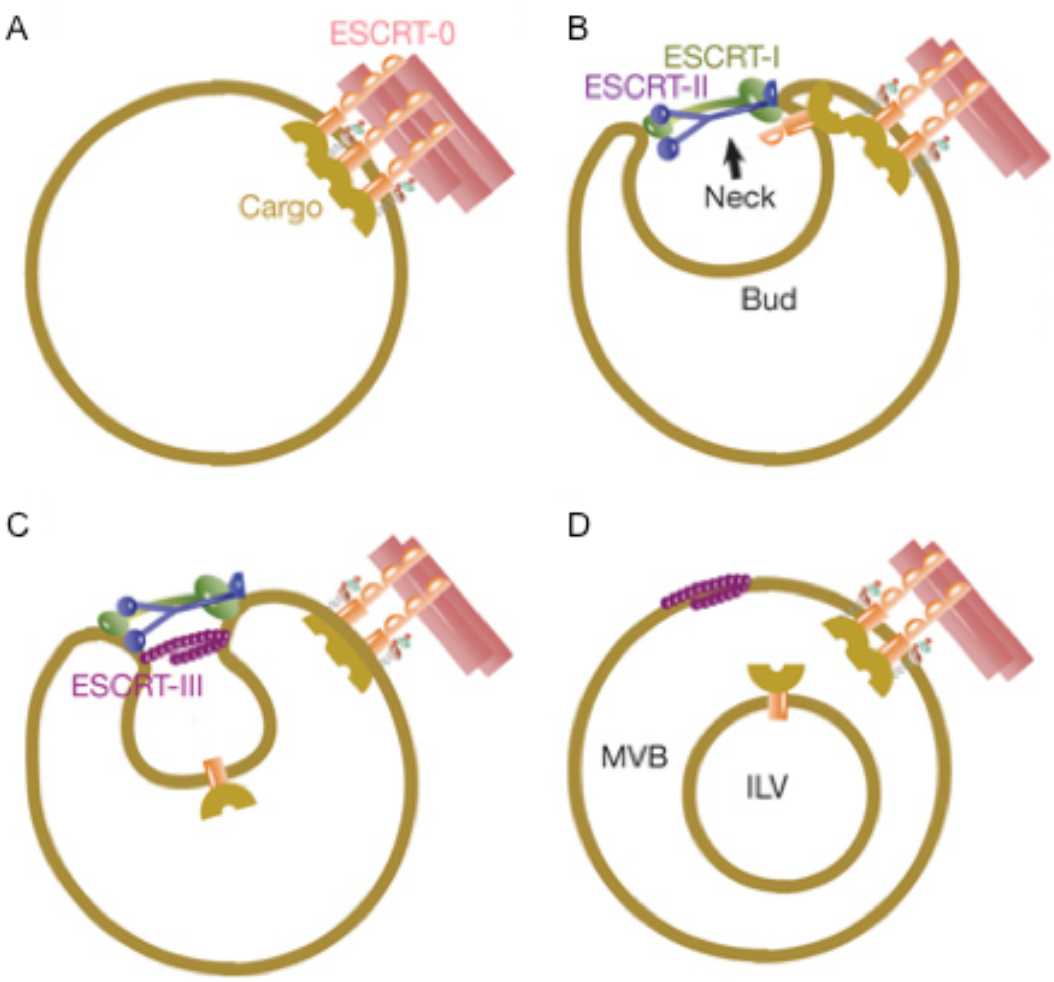


Figure 6. Molecular model for MVB biogenesis. (A) ESCRT-0 self-assembles and clusters ubiquitinated cargos on endosomal membranes. (B) ESCRT-I and -II deform the membrane into a bud and stabilize the bud neck. ESCRT-I recruits ESCRT-0, facilitating deposition of cargo into the bud. (C) ESCRT-II nucleates assembly of ESCRT-III components in the bud neck into an interior collar. (D) ESCRT-III mediates membrane scission to release the nascent vesicle into the endosome lumen. ESCRT components remain on the outside of the limiting membrane until they are recycled by Vps4. Taken from Wollert and Hurley, *Nature*, 2010 [185].

The ESCRT pathway mediates multiple topologically similar membrane scission events

Recently, the ESCRT pathway has been implicated in mediating the plasma membrane abscission event of cytokinesis, required for separation of daughter cells from each other. Tsg101 and ALIX localize to the midbody, a constricted membrane structure between the daughter cells, and their interaction with Cep55 is essential for the resolution of the membrane tether [186,187]. Tsg101 binding to ESCRT-I component Vps28 [186] and ALIX-mediated recruitment of ESCRT-III proteins to the midbody are also important for cytokinesis [187,188]. Moreover, overexpression of ESCRT-III proteins or catalytically inactive Vps4 inhibits cytokinesis [186,187,189]. Importantly, the role of ESCRT proteins in cell division appears to be direct and independent of endosomal protein sorting, since catalytic activity of Vps4 is required for cytokinesis in archaea, a separate domain of life that lacks endosomal membranes [190,191].

The Class E VPS machinery executes a membrane fission event common to three functionally distinct but topologically equivalent processes (Fig. 7). The membrane deformation activity of ESCRT proteins is commonly thought to be dispensable for viral budding, since Gag multimerization on membranes appears to have an intrinsic capacity to induce membrane curvature [192]. However, late domain mutation in some retroviruses, including Mason-Pfizer monkey virus (MPMV) and murine leukemia virus (MLV), has been demonstrated to induce early budding defects, manifested by aberrant or inefficient assembly [110,193,194].

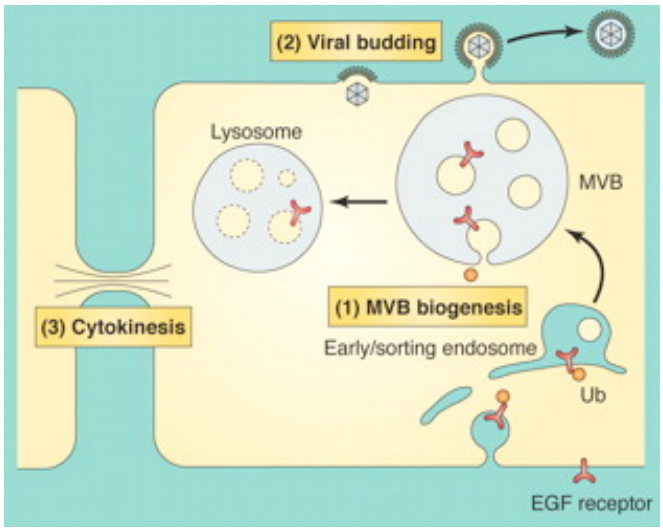


Figure 7. The ESCRT machinery mediates three topologically similar membrane scission events: (1) budding of vesicles into the lumen of multivesicular bodies, (2) late domain-dependent separation of nascent enveloped virions from the host cell, and (3) abscission of the plasma membrane between two daughter cells during cytokinesis. Taken from Wollert, et. al., *J. Cell Science*, 2009 [195].

As previously mentioned, retroviruses rely on cis-acting elements, called late domains, within their structural Gag proteins to co-opt the cellular vacuolar protein sorting pathway. Inhibition of the Class E pathway by expression of dominant negative Vps4 or ESCRT-III components potently blocks the late stage of virion production by retroviruses encoding diverse late domains [103,108,113,115,122-124,196]. Viruses containing YPxL- or LxxLF-type late domains, such as EIAV, engage the Class E pathway by recruiting ALIX [108], which can in turn recruit the core membrane scission machinery of ESCRT-III through its direct interaction with CHMP4 proteins [197]. Indeed, the Bro1 domain of ALIX, which mediates this interaction, is essential for its ability to mediate late domain-dependent budding [196,198]. Viruses encoding PT/SAP-type late domains, including HIV-1, recruit Tsg101 [103,113,114], a core component of ESCRT-I, which can recruit ESCRT-III indirectly through interactions with ALIX

[108,122,123,196] or ESCRT-II [123,150,167,168]. Moreover, HIV-1 has been reported to specifically package Tsg101, Vps28, ALIX, and Vps4 inside virions [108,123]. In contrast, viruses encoding PPxY-type late domains, such as murine leukemia virus (MLV) and Rous sarcoma virus (RSV), recruit Nedd4-like HECT ubiquitin E3 ligases, which have no known binding partners in the Class E pathway [111,115-121].

HECT ubiquitin ligase biochemistry and functions

HECT ligases play an important role in directing the substrate specificity of the cellular ubiquitylation cascade. Ubiquitin conjugation is a multistep process, involving the sequential transfer of ubiquitin from an E1 activating enzyme to E2 conjugating enzymes, then to E3 ligating enzymes, and finally to substrate proteins (reviewed in [129]). The E1 enzyme initiates the cascade by adenylating the carboxy terminus of ubiquitin with ATP and then attacking the resulting high-energy anhydride bond with the sulfhydryl group of its catalytic cysteine to generate an E1-Ub intermediate. The E2 enzyme, in turn, attacks the high-energy thioester bond linking ubiquitin to the E1 enzyme using its own catalytic cysteine, thus transferring the ubiquitin moiety to its own active site. Finally, the E3 ligase promotes the transfer of ubiquitin from the E2 to a specific substrate. Eukaryotes express two classes of E3 ubiquitin-protein ligases that function via distinct mechanisms. RING family E3 ligases lack intrinsic enzymatic activity and function primarily as scaffolds that position the reactive E2-Ub thioester bond in close proximity to the nucleophilic amino group of the target lysine residue on the substrate protein [199]. In addition, RING ligases may trigger conformational changes in the E2 to stimulate ubiquitin release, thus facilitating its transfer [200]. In

contrast, the active site of HECT family E3 ligases contains an essential cysteine residue that forms a direct thioester bond with ubiquitin before catalyzing its transfer to the substrate [201].

Humans encode 28 known HECT ubiquitin E3 ligases [202], a subset of which has been grouped into two families based on shared domain organization: the Nedd4 family contains nine members (Fig. 8B), and the HERC family contains six members. Nedd4-like HECT ligases contain an N-terminal C2 domain, two to four WW domains, and a C-terminal HECT domain (Fig. 8A). The C2 domain binds phosphoinositides and mediates ligase recruitment to the plasma membrane and endosomal membranes [203,204]. The WW domains contain WxxP repeats that bind PPxY motifs in substrate and adapter proteins [205]. The HECT domain mediates catalysis, using a conserved cysteine residue near its C-terminus [201,206]. HERC family members contain a catalytic HECT domain and one or more regulator of chromosome condensation 1 (RCC1)-like domains (RLDs), which bind chromatin and may possess guanine nucleotide-exchange factor (GEF) activity [207,208]. The remaining HECT ligases possess various domains in addition to HECT, including zinc fingers, UBA domains, ankryn repeats, and RING fingers [209]. HECT ligases perform important regulatory roles in diverse cellular processes, such as endocytosis, endosomal trafficking, chromatin remodeling, and gene expression, and their malfunction has been associated with numerous physiological disorders, including cancers, autoimmunity, hypertension, and developmental defects (reviewed in [209]).

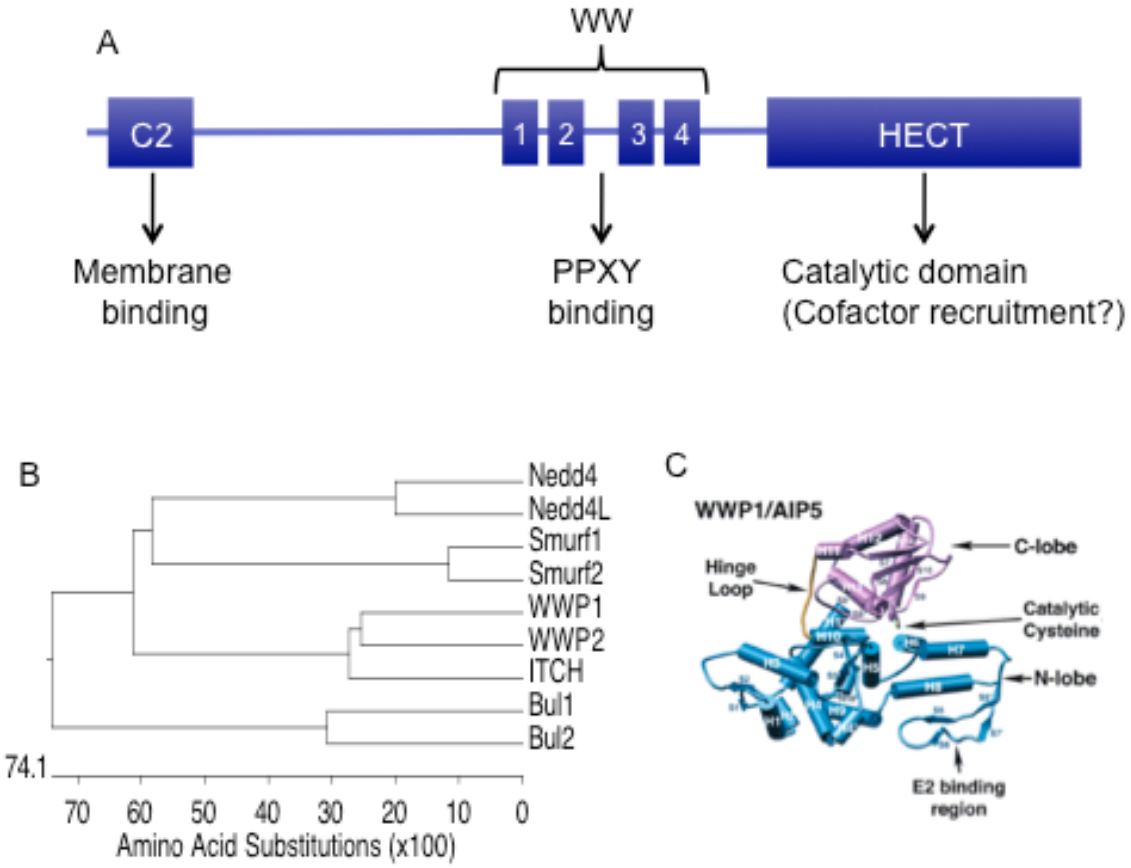


Figure 8. Nedd4-like HECT ubiquitin E3 ligases. (A) Typical domain organization of Nedd4 family members. (B) Phylogenetic tree of the nine human Nedd4-like HECT ligases. (C) Structure of the HECT domain of WWP1. The N-lobe (blue, residues 546–802) is connected to the C-lobe (magenta, residues 546-802) by a short flexible hinge loop (residues 803–806). Taken from Verdecia*, Molecular Cell*, 2003 [210].

The catalytic domains of HECT ligases contain two distinct polypeptide lobes connected by a hinge loop (Fig. 8C). The elongated N-lobe binds E2 enzymes, while the smaller C-lobe contains the residues critical for catalysis. Hinge loop flexibility is required for catalytic activity, presumably because the C-lobe must rotate with respect to the N-lobe, in order to align its catalytic cysteine either with the E2-bound ubiquitin or with the lysine acceptor residue on the substrate [210]. A highly conserved phenylalanine residue near the C-terminus of HECT domains is dispensable for thioester bond formation between ubiquitin and the catalytic cysteine but essential for the subsequent ubiquitin transfer to the substrate [211,212]. Several HECT domains possess a non-covalent ubiquitin-binding site in the N-lobe, which appears to regulate the length of and, possibly, linkage types in the synthesized ubiquitin chains. The ubiquitin-interacting site of mammalian Smurf2 HECT contributes to efficient binding of monoubiquitinated substrates and is required for polyubiquitination [213]. In contrast, the analogous binding site in yeast Rsp5 HECT seems to limit ubiquitin chain elongation [214].

Although isolated HECT domains are thought to be constitutively active, HECT ligases have evolved various mechanisms to regulate their catalytic activity. These mechanisms limit HECT ligase promiscuity and protect the ligases themselves from excessive autoubiquitination and consequent degradation. For example, intramolecular interactions between the C2 and HECT domains of Smurf2 inhibit its activity by obstructing the catalytic cysteine. Similar autoinhibitory interactions have been observed in WWP2 and Nedd4 [212]. The Smad7 adapter protein relieves Smurf2 autoinhibition by displacing the C2 domain from the N-lobe of the HECT domain and further stimulates catalytic activity by recruiting the UbcH7 E2 enzyme [212,215]. Itch and Nedd4L ligases

are autoinhibited by intramolecular interactions between their WW and HECT domains [216,217]. JNK1 kinase relieves Itch autoinhibition by phosphorylating the proline-rich region of the HECT domain, thereby destabilizing its association with the WW domains [217]. Autoinhibition of Nedd4L is likely relieved by substrate binding to its WW domains [216]. Deubiquitinating enzymes can antagonize the activity of some processive HECT ligases, such as yeast Rsp5, by partially disassembling their ubiquitin chains to produce mono- and oligo-ubiquitinated substrates [218]. In addition HECT ligase activity can be both positively and negatively regulated at the substrate recognition level by adaptors that facilitate or compete with substrate binding to WW domains [219-224].

The yeast HECT ligase Rsp5 has been implicated in mediating both ubiquitin-dependent and -independent cargo sorting into the MVB pathway. Rsp5 expression is dispensable for MVB biogenesis, but its deletion is associated with reduction in the average size of intralumenal vesicles [225]. Direct interaction of the endosomal cargo protein Sna3 with Rsp5 via an essential PPAY motif is required for its delivery to the vacuole; however, ubiquitination of Sna3 appears to be dispensable for its sorting [225-227]. Remarkably, the catalytic activity of Rsp5 is essential for efficient sorting of lysine-free Sna3, which does not become ubiquitinated [226,227]. Therefore, Rsp5 may promote MVB sorting of non-ubiquitinated cargo by ubiquitinating either itself or unknown adaptor proteins.

HECT ligases and ubiquitin in retroviral budding

The catalytic activity of several HECT ubiquitin ligases is essential for their ability to promote PPxY-dependent virus budding [115,120]. Additionally,

overexpression of the ubiquitin-like molecule interferon-stimulated gene 15 (ISG15) has been reported to suppress PPxY-dependent Ebola VLP production by inhibiting the catalytic activity of Nedd4 [228,229]. Overexpression of catalytically inactive HECT ligases inhibits PPxY-dependent budding of murine leukemia virus (MLV), MPMV, and Ebola virus [115,120]. Truncated WWP1, lacking the entire catalytic domain, inhibits MLV budding more potently than full length WWP1 with a disrupted active site [115], suggesting that HECT domains may possess activities other than ubiquitin conjugation, such as cofactor recruitment, that are important for their function in viral budding. Consistent with this notion, HECT domains localize to membranes of aberrant 'Class E' compartments induced by overexpression of catalytically inactive ATPase Vps4 [115]. Since many VPS factors are trapped on Vps4-induced compartments, HECT domains may be recruited to these membranes by interaction with VPS proteins, either directly or through unidentified bridging factors. Interestingly, two recent studies have demonstrated significant stimulation of virion production by HIV-1 Gag lacking known late domains by overexpression of the HECT ligase Nedd4L. The rescue required the catalytic activity of Nedd4L, expression of Tsg101, and an intact Class E pathway and was accompanied by an increase in the ubiquitination of ESCRT-I components [230,231]. However, direct association between HECT ligases and ESCRT proteins has not been demonstrated.

Multiple lines of evidence suggest a more general role of ubiquitin in retroviral budding. For example, free ubiquitin is enriched in retrovirus particles [232,233], and low levels of mono or oligo-ubiquitinated Gag have been detected in HIV-1 virions [234]. Furthermore, proteasome inhibitors block the release of retroviruses that encode PT/SAP or PPxY late domains, most likely by depleting free ubiquitin from cells

[104,116,235,236]. Since ubiquitination of cellular proteins can serve as a signal for MVB sorting by recruiting ESCRT proteins, deposition of ubiquitin on viral structural proteins may also lead to engagement of the class E machinery. Consistent with this notion, fusion of ubiquitin to the PTAP-encoding late domain of HIV-1 Gag significantly enhances its affinity for the Tsg101 UEV domain, despite the inherently weak affinity of the UEV domain for monoubiquitin alone [103]. Moreover, mutation of multiple ubiquitin acceptor lysine residues in Gag has been shown to inhibit particle production by several retroviruses [237,238].

To address whether ubiquitination of viral proteins is generally required for enveloped viral particle release we took advantage of an unusual retroviral Gag protein that is almost devoid of lysines. We found that VLP release proceeded efficiently in the absence of ubiquitin acceptors in the viral protein, when dependent on a motif (PSAP) that directly recruits the ESCRT machinery. Remarkably, when particle release was engineered to be dependent on a PPxY motif and a catalytically active ubiquitin ligase, ubiquitin acceptors in the viral protein remained dispensable. Thus, ubiquitin-dependent release of enveloped viral particles appears to involve ubiquitin conjugation to trans-acting factors rather than directly to viral proteins. We also showed that the catalytic activity of different HECT domains is essential for their ability to promote PPxY-dependent VLP release. However, stimulation of budding correlated directly with autoubiquitination of HECT domains rather than their ability to ubiquitinate Gag. Moreover, direct fusion of a single ubiquitin moiety to the C-terminus of PFV Gag was sufficient to promote both late domain-dependent and -independent budding. The ability of ubiquitin to effectively compensate for the lack of conventional late domain function

required the Ile44 hydrophobic patch of ubiquitin and an intact cellular VPS pathway. Our results support a model for HECT ligase-mediated viral budding, whereby the stimulation of protein ubiquitination in the vicinity of VLP assembly promotes the concentration of ubiquitin-interacting Class E proteins and consequent recruitment of the ESCRT machinery to nascent virions.

Chapter II. Materials and Methods

Construction of membrane-targeted PFV Gag expression plasmids

The PFVΔEnv proviral plasmid and the PFV Env expression plasmid have been described previously, and were a gift from A. Rethwilm [239]. The wild-type and mutant ($PSAP_{284-287}AAAT$) PFV Gag open reading frames were amplified from corresponding proviral PFV plasmids, that have been previously described [240], using primers directed to the 5' and 3' termini of the PFV Gag coding sequence and appended with NcoI and XhoI restriction enzyme sites, respectively. These amplicons were inserted into an NcoI/XhoI digested pCAGGS expression vector, to generate pCAGGS/Gag and pCAGGS/Gag(PTAP-) respectively. Membrane targeted Gag expression plasmids were derived from pCAGGS/Gag by inserting annealed oligonucleotides encoding the peptides MyrR (MGARASGSGRRRGSGRRR), Fyn (MGCVQCKDKE), FynR (MGCVQCRDRE), and Lck (MGCGCSSHPE) into the NcoI site at the 5' end of PFV Gag. Lysine-free PFV Gag proteins were generated by Lys396 to Arg by PCR based mutagenesis. The pCAGGS/Gag(ASAP) expression plasmid was derived from pCAGGS/Gag by PCR based mutagenesis. pCAGGS plasmids expressing Lck-Gag-PY, Lck-Gag-PY-3K, or Lck-Gag-PSAP-PY were constructed by inserting the MLV late domain with or without three appended lysine residues (LLTEDPPPYRD [KKK]) at the 3' end of Lck-Gag(K396R) using long PCR primers directed to the C-terminus of Gag. Plasmids expressing cyan-fluorescent fusions of PFV Gag proteins were constructed by inserting cerulean fluorescent protein encoding sequence into the 3' XhoI site of all pCAGGS/PFVGag derivatives. pCAGGS/Gag-UbΔGG expression plasmids were

generated by overlap PCR, using pCAGGs/Gag(PSAP), pCAGGS/Gag(PSAP-), and pCAGGS/Gag(PSAP-PY) as templates for the N-terminal portions and pHA-ubiquitin as the template for the C-terminal portion. The K48R, K63R, F4A, L8A, I44A, and QE62,64AA point mutations were introduced into Lck-PFV Gag-PSAP-Ub and Lck-PFV Gag-ΔPSAP-Ub constructs by PCR-based mutagenesis. Each cDNA was cloned into pCAGGs for expression in mammalian cells.

Construction of chimeric HECT ligase expression plasmids

Plasmids expressing WWP1, WWP2, Nedd4, Nedd4L, Itch, Smurf1, and Rsp5 have been described previously [115,225]. The Bul2 HECT domain (encoding residues 1189-1572) was PCR amplified from 293T cell cDNA. Chimeric HECT ligases containing the C2 and WW domains of WWP1 and heterologous HECT domains were constructed by overlap PCR and cloned into the pCR3.1/YFP expression vector, fused to the C-terminus of YFP, as outlined in Table 2. Plasmids expressing catalytically inactive WWP1 (C890S) and Rsp5 (C777S) have been described previously [115,225], and Rsp5 (C777S) was used as a template to generate the catalytically inactive WWP1/Rsp5 expression plasmid. Plasmids expressing catalytically inactive chimeric ligases were generated by introducing the following point mutations into wild type chimeric ligases by PCR mutagenesis: WWP2 C838S, Nedd4 C867S, Nedd4L C922S, Itch C830S, Smurf1 C725S, Bul2 C1540S.

Table 2. Chimeric HECT ligase construction.

Construct	N-fragment	C-fragment	Junction primers	Cloning sites
WWP1/WWP2	WWP1 1-542	WWP2 491-870	GGTGGTCCACAAATTGCTTATGACCGCAGTTTTCGGTGGAAG CCGAAAACTGCGGTCATAAGCAATTTGTGGACCACCTTTAG	EcoRI/NotI
WWP1/Nedd4	WWP1 1-542	Nedd4 520-902	GGTGGTCCACAAATTGCTTACTCCAGGGATTACAAAAGAAAG CTTTTGTAATCCCTGGAGTAAGCAATTTGTGGACCACCTTTAG	EcoRI
WWP1/Nedd4L	WWP1 1-542	Nedd4L 593-975	GGTGGTCCACAAATTGCTTACTCCAGAGAATTTAAGCAGAAATATG CTTAAATTCTCTGGAGTAAGCAATTTGTGGACCACCTTTAG	EcoRI/NotI
WWP1/Itch	WWP1 1-542	Itch 483-862	GGTGGTCCACAAATTGCTTATGTTCGGGACTTCAAAGCAAAG CTTTGAAGTCCCGAACATAAGCAATTTGTGGACCACCTTTAG	EcoRI/NotI
WWP1/Smurf1	WWP1 1-542	Smurf1 374-757	GGTGGTCCACAAATTGCTTACGAAAGAGATCTAGTCCAGAAG GACTAGATCTCTTTCGTAAGCAATTTGTGGACCACCTTTAG	EcoRI/NotI
WWP1/Bul2	WWP1 1-542	Bul2 1189-1572	GGTGGTCCACAAATTGCTTACAAGCGGGATTTCGAAGCC CTTCGAAATCCCGCTTGTAAGCAATTTGTGGACCACCTTTAG	XhoI/NotI
WWP1/Rsp5	WWP1 1-542	Rsp5 431-809	GGTGGTCCACAAATTGCTTACAAGCGTGACTTCAGACGTAAG GTCTGAAGTCACGCTTGTAAGCAATTTGTGGACCACCTTTAG	EcoRI/XhoI

Construction of chimeric Tsg101/HECT expression plasmids

The Tsg101 expression plasmid has been described previously [113,241]. Tsg-HECT and WWP1-TsgΔUEV chimeras were generated by overlap PCR, using full length Tsg101, WWP1, Nedd4L, and Itch as templates, as outlined in Table 3. The amplicons were then cloned into EcoRI/NotI-digested pCR3.1/YFP expression plasmids, fused to the C-terminus of YFP. Point mutations to inactivate the catalytic domain of each Tsg-HECT construct (described above) were introduced by PCR-based mutagenesis.

Table 3. Construction of Tsg101/HECT chimeras

Construct	N-fragment	C-fragment	Junction primers
Tsg-WWP1	Tsg101 1-157	WWP1 543-922	CCGCCATACCAGGCAACGTATGAACGCGGCTTTAGGTGGAAGC CCTAAAGCCGCGTTCATACGTTGCCTGGTATGGCGGATAGG
Tsg-Nedd4L	Tsg101 1-157	Nedd4L 593-975	CCGCCATACCAGGCAACGTACTCCAGAGAATTTAAGCAGAAATATG CTTAAATTCTCTGGAGTACGTTGCCTGGTATGGCGGATAGG
Tsg-Itch	Tsg101 1-157	Itch 483-862	CCGCCATACCAGGCAACGTATGTTCGGGACTTCAAAGCAAAG CTTTGAAGTCCCGAACATACGTTGCCTGGTATGGCGGATAGG
WWP1-TsgΔUEV	WWP1 1-542	Tsg101 157-390	GGTGGTCCACAAATTGCTGGGCCACCAAATACTTCCTACATGC GGAAGTATTTGGTGGCCCAGCAATTTGTGGACCACCTTTAG

Additional expression plasmids

The HIV-1 HXB ΔPTAP proviral plasmid has been described previously [113]. Yeast two-hybrid plasmids encoding wild type and I44A ubiquitin were constructed by PCR amplification of ubiquitinΔGG from the pHA-ubiquitin plasmid using 5' and 3' primers appended with EcoRI restriction sites and cloning into the pGBKT7 (Clontech) and pVP16 vectors [113]. The Class E factor yeast two-hybrid library, encoding a panel of Class E VPS factors in pGBKT7 and pVP16 backgrounds, has been described [122]. pCR3.1-based expression plasmids encoding fluorescent fusions of WWP1ΔHECT, Vps4 E228Q, Tsg101UEV, Hrs, ALIX, UBPY, and Eap45 have been described previously [113,115,122,241]. The following mutants, impaired for ubiquitin binding, were generated by PCR mutagenesis: Tsg101UEV N45A, Hrs LA265,266AL, and Eap45 VFE67,68,70AAA.

Cell lines and transfections

293T and HeLa cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and 10μg/ml gentamycin (Gibco). For 293T cell transfections, plasmids were mixed in 100μl serum-free DMEM, followed by addition of 4μg PEI (Polyethylenimine; Polysciences) per 1μg total DNA (from a 1mg/ml PEI stock solution). This mixture was vortexed briefly and incubated at room temperature for 15 minutes before addition to cells. Transfections were allowed to proceed for 4 hours before replacing with fresh media. For HeLa cell transfections, plasmids were mixed in 100μl serum-free DMEM and vortexed briefly. A master mix containing 100μl serum-free DMEM and 5μl Lipofectamine 2000 (Invitrogen) per reaction was prepared, mixed gently by pipetting, and incubated at room temperature for 5 minutes. The Lipofectamine 2000 mixture (105μl) was then added to the plasmid mixture and incubated at room temperature for 15 minutes before addition to cells. Transfections were allowed to proceed for 4 hours before replacing with fresh media.

Western Blot Analysis

Virion and cell lysates and immunoprecipitates were separated on polyacrylamide gels, transferred to nitrocellulose membranes, and probed with various antibodies: anti-PFV human serum, anti-HIV-1 p24CA (183-H12-5C), anti-EIAV equine serum (VMRD, Inc.), anti-GFP (Roche), and anti-HA (HA.11, Covance). Subsequently, the blots were probed with species-specific peroxidase-conjugated secondary antibodies and chemiluminescent substrate reagents (Pierce) for conventional Western blotting. Alternatively, membranes

were probed with species-specific antibodies conjugated to IRDye800CW, and fluorescent signals were detected and quantified using a LICOR Odyssey scanner.

Virus particle release assay

PFV Gag VLP release assays were performed either in 100mm plate or 6-well plate formats. In 100mm plates, 293T cells were seeded at 3×10^6 cells per plate and transfected the following day with 2μg pCAGGS/Gag plasmids, alone or with 4μg pCR3.1/YFP, pCR3.1/YFP-WWP1, or a derivative. Virus-like particles were pelleted by ultracentrifugation of 8ml 0.22-μm-filtered culture supernatants, collected 24 hours post-transfection, over a 4ml 20% sucrose cushion for 2 hours at 100,000 × g. In 6-well plates, 293T cells were seeded at 5×10^5 293T cells per well and transfected the following day with 1μg of pCAGGs/PFVGag-derived plasmids, alone or with 1μg of pCR3.1/YFP, pCR3.1/YFP-WWP1-HECT, pCR3.1/YFP-TsgUEV-HECT, or pCR3.1/YFP-WWP1-TsgΔUEV plasmids. Alternatively cells were transfected with 1μg of pCAGGs/PFVGag-derived plasmids and the indicated amounts of fluorescent Class E factor expression plasmids. Virus-like particles were pelleted by ultracentrifugation of 2ml 0.22-μm-filtered culture supernatants, collected 48 hours post-transfection, over a 2ml 20% sucrose cushion for 90 min at 115,000 × g.

HIV-1 and EIAV VLP release assays were performed in 6-well plates. 293T cells were seeded at 5×10^5 293T cells per well and transfected with 500ng of pCR3.1/HIV-1Gag or pCR3.1/EIAVGag expression plasmids or HIV-1 HXB-ΔPSAP proviral plasmid and the indicated amounts of fluorescent Class E factor expression plasmids. VLPs were pelleted by ultracentrifugation of 2ml of 0.22-μm-filtered culture supernatants, collected

48 hours after transfection, over a 2-ml 20% sucrose cushion for 90 min at 115,000 × g. VLP and cell lysates were analyzed by conventional or quantitative Western blotting.

Ubiquitin transfer assay

293T cells (5×10^5) in six-well plates were cotransfected with 1 μg of pCAGGs/PFVGag-PY-3K, 500 ng of pHA-ubiquitin, and 1 μg of the indicated chimeric pCR3.1-WWP1-HECT ligases. At 36 hours after transfection, each well of cells was thoroughly lysed at room temperature with 400μl of denaturing lysis buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 1.0% glycerol, 0.5% SDS, supplemented with protease inhibitor tablets (Roche) and 5mM N-ethylmaleimide to inhibit deubiquitination), sonicated, and cleared of cellular debris by microcentrifugation at 14,000 rpm for 10min. The lysates were then diluted 5-fold, to adjust the concentration of SDS to 0.1% and NP-40 to 1.0%, and split into two fractions. From one fraction, Gag proteins were immunoprecipitated with αPFV serum and protein G-Sepharose beads. From the other fraction, YFP-HECT ligase proteins were immunoprecipitated with αGFP monoclonal antibody and protein G-Sepharose beads. The beads were washed three times with RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.1% SDS and 1.0% NP-40), and proteins were eluted from the beads by boiling for 10 minutes in 2x SDS protein sample buffer, supplemented with 2% 2-mercaptoethanol. Immunoprecipitates and unfractionated cell lysates were then analyzed by conventional chemiluminescent or quantitative fluorescent Western blotting.

Fluorescence microscopy

HeLa cells (4×10^5) on 35-mm poly-D-lysine coated glass bottom dishes (Mattek) were cotransfected with plasmids expressing CFP-fused and unfused Lck-Gag at a 1:1 ratio, either alone or along with 200 ng pCR3.1/YFP-WWP1-WW. Twenty-four hours post transfection cells were fixed in paraformaldehyde and visualized by deconvolution microscopy using an Olympus IX70-based DeltaVision system (Applied Precision) equipped with a 100× magnification oil immersion objective and a Photometrics CH350L camera. Optical filters were used to distinguish YFP and CFP fluorophores. For each condition, a stack of 30 images spaced by 0.3μm in the Z-dimension was acquired and deconvolved using SoftWorx software (Applied Precision), and single optical sections at the center of the cell and at the cell-coverslip interface were selected.

Electron microscopy

Electron micrographs of assembling Lck-PFV virus-like particles were generated by Marc C. Johnson (Department of Molecular Microbiology and Immunology, University of Missouri). Briefly, DF-1 cells were transfected with plasmids expressing GFP-VPS4 (E228K) along with Lck-Gag, or MLV GagPol along with pdsRED, and fixed in paraformaldehyde. After fluorescence imaging, cells were fixed in glutaraldehyde, postfixed in osmium tetroxide, dehydrated in ethanol, critical point-dried, and sputter-coated with gold palladium. Cells were identified by their location on a finder grid and imaged by using a LEO 1550 field-emission scanning electron microscope.

Yeast two-hybrid assay

Yeast cells (Y190) were transformed with 1μg each of the pGBKT7- and pVP16-derived plasmids described above. Cells transformed with pGBKT7 plasmids expressing each class E factor were co-transformed with either empty pVP16 vector, pVP16-Ub or pVP16-Ub(I44A). Alternatively, cells transformed with pVP16 plasmids expressing each class E factor were co-transformed with either empty pGBKT7 vector, PGBKT7-Ub, or pGBKT7-Ub(I44A). Double transformants were selected on supplemented agar plates lacking leucine and tryptophan. After two days, yeast pellets consisting of 10-20 colonies were resuspended in 0.5ml Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, 0.28% 2-mercaptoethanol, 0.01% SDS) and permeabilized by addition of 25μl chloroform and vortexing. Protein-protein interactions were assayed by β-galactosidase reporter activity. The β-galactosidase substrate chlorophenol red-β-D-galactopyranoside (CPRG, Roche) was added to a final concentration of 1.4mg/ml, and the suspensions were mixed by vortexing and incubated at room temperature for 30 minutes. The optical density of the supernatants at 540nm was measured after pelleting the cell debris by microcentrifugation,

RNA interference

293T cells were transfected in 6-well plates using a modified Lipofectamine 2000 protocol. Cells were seeded at 3×10^5 cells per well in DMEM supplemented with 10% fetal bovine serum containing no antibiotics and transfected the following day with siGENOME siRNAs targeting Luciferase, Tsg101, Hrs, Alix, UBPY, or Eap45 (Dharmacon). For single siRNA transfections, 40pmol siRNA was combined with 200ml

serum-free OptiMEM and a master mix consisting of 6µl Lipofectamine 2000 and 200µl OptiMEM per reaction was prepared. After incubating both mixtures at room temperature for 15 minutes, 206µl of the Lipofectamine 2000 mixture was added to each siRNA mixture and mixed gently by pipetting. After another 15-minute incubation at room temperature, the reactions were added to cells. Transfections were allowed to proceed for 4 hours before replacing with fresh media. After 24 hours, cells were transfected again with the same siRNAs and the indicated Gag expression plasmids. VLP and cell lysates were prepared 48 hours after the second transfection. For combination knockdown experiments, 293T cells were transfected with 40 pmol of each siRNA, and the total amounts of siRNA tranfected were maintained at 80 pmol per reaction. To assess knockdown efficiency, 293T cells were transfected once with YFP-Tsg101, -Hrs, -ALIX, -UBPY, or -Eap45 expression plasmids and corresponding siRNAs. GFP expression in cell lysates harvested 48 hours after transfection was assayed by quantitative Western blotting.

Chapter III. Direct viral protein ubiquitination is dispensable for ubiquitin ligase-dependent virus particle budding

The Gag proteins of several retroviruses carry low levels of ubiquitin [232,233,234], and the extent of ubiquitinaton appears to depend on the type of late domain they encode [112]. However, the functional relevance of direct Gag ubiquitination is disputed. On the one hand, mutation of multiple lysine residues in Gag blocks the late phase of HIV-1 and RSV budding at the plasma membrane without significantly affecting particle morphology [237,238]. On the other hand, budding efficiency does not necessarily correlate with the extent of Gag ubiquitination, as transplantation of PPxY late domains into late domain-deficient HIV-1 Gag leads to an increase in Gag ubiquitination but fails to rescue particle production [112]. Additionally, the inhibition of HIV-1 and RSV budding by extensive mutagenesis of Gag lysines may result from disruption of structural elements important for the late stage of particle release rather than the loss of ubiquitin acceptor sites. We noticed that the Gag proteins of the Spumaviruses (or Foamy viruses) are remarkably lysine-poor. This is an extremely unusual property, shared by all known members of the spumavirus subfamily (Table 4), to test whether ubiquitination of viral proteins is essential for the activity of late domains.

Table 4. Abundance of basic residues in retroviral Gag proteins.

Viral Gag	HIV-1	MLV	RSV	MPMV	PFV	SFV-1	SFVcpz	SFVora	FeFV	BFV	EqFV
Lys	38	31	30	58	1	0	2	1	8	0	1
Arg	28	50	48	22	64	56	61	60	46	48	50

Spumaviruses comprise a unique subfamily of retroviruses (Fig. 9) with several distinguishing characteristics (reviewed in [242,243]). Prototype foamy virus (PFV) utilizes a dual promoter system for transcription. The internal promoter, which drives the expression of Tas and Bet accessory genes, exhibits higher basal activity than the LTR promoter. Tas encodes the viral transactivator protein, which enhances transcription from both the LTR and the internal promoter. Unlike orthoretroviruses, which express Pol as part of a Gag-Pol polyprotein, PFV expresses Pol from a separately spliced mRNA. The precise mechanism of Pol incorporation into virions is unclear but appears to require Pol interaction with Gag, either direct or through co-association of both proteins with the viral genomic RNA [244,245]. PFV Gag lacks the canonical Cys-His boxes required for RNA binding of orthoretroviral NC proteins. Instead, three glycine- and arginine-rich repeat regions (GR boxes) mediate Gag interaction with nucleic acids (both RNA and DNA) and nuclear import. Although the viral genome is packaged as RNA, reverse transcription occurs late in the viral lifecycle, before or during budding [246]. Therefore, the major form of infectious nucleic acid in the PFV virions appears to be DNA [247].

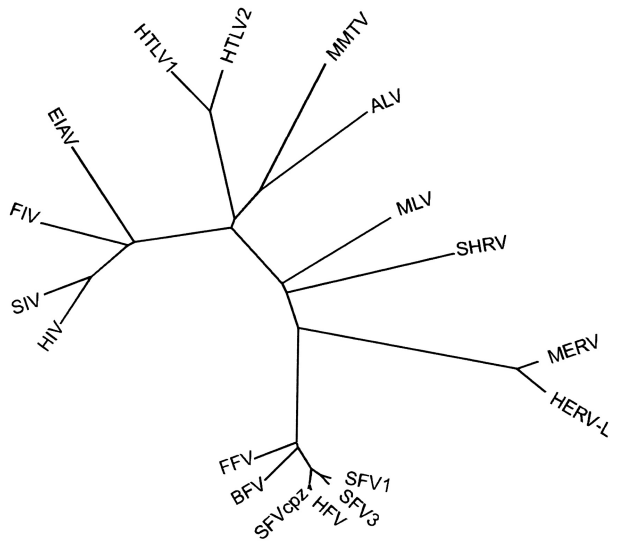


Figure 9. Phylogenetic tree of retroviral *pol* sequences. The tree was derived by alignment of ungapped portions of Pol protein sequences. Taken from [243].

The Gag protein of Foamy viruses does not undergo conventional processing into mature matrix (MA), capsid (CA), and nucleocapsid (NC) products. Instead the full-length 72-kDa precursor is cleaved near its C-terminus into 68- and 4-kDa products, and the two high molecular weight proteins are detected in virions at approximately equimolar amounts [248]. PFV encodes PT/SAP as its primary L-domain (Fig. 10A), which induces viral particle release by binding to the Tsg101 component of ESCRT-I [240,249]. While PFV Gag only contains a single lysine (residue 396, Fig. 10A), there are potential ways in which the near-absence of lysine acceptors in FV Gag proteins could be circumvented while maintaining a requirement for viral protein ubiquitination in particle release. For example, unlike many retroviral Gag proteins, the N-terminus of PFV Gag is not myristoylated and could serve as a putative ubiquitin acceptor site. Additionally, FV Pol proteins do not exhibit the striking lysine-poor sequence bias of Gag and provide numerous opportunities for virion protein ubiquitination. Finally, FVs are unusual among retroviruses in that they pre-assemble complete capsids in the cytoplasm of infected cells and bud preferentially at intracytoplasmic membranes into vesicles, which are thought to be subsequently transported to the cell surface [250,251]. The leader peptide of the cognate Env protein is required to direct FV capsids to membranes for envelopment and release [248,252]. Notably, the cytoplasmic portion of the PFV Env leader peptide contains five lysine residues, which are extensively ubiquitinated [253]. Thus, virion proteins other than Gag could substitute for the presumed role of Gag ubiquitination in virion release. Therefore, we first derived an experimental system in which extracellular PFV VLPs were generated by Gag in the absence of other viral proteins.

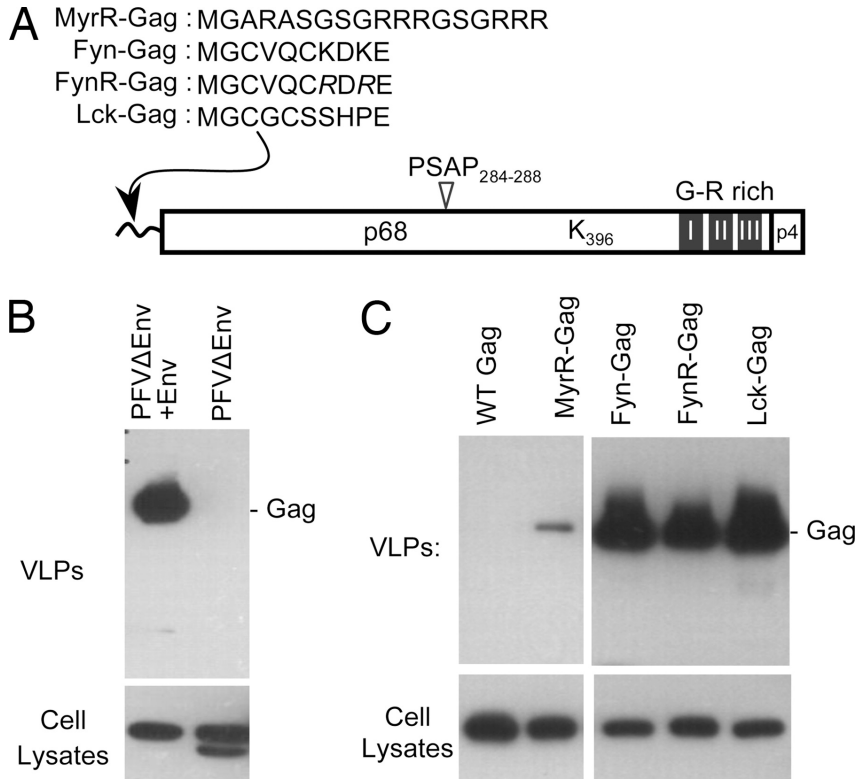


Figure 10. Envelope-independent release of VLPs generated by membrane-targeted PFV Gag proteins. (A) Schematic representation of the PFV Gag protein, indicating relevant landmarks [the PSAP late domain, a single lysine residue, glycine/arginine (G-R)-rich domains, and a proteolytic cleavage site near the C-terminus of the ~72-kDa protein]. Also shown are the various membrane-targeting peptides that were appended to the PFV Gag N terminus. (B) Virion production by 293T cells transfected with the PFVΔEnv proviral plasmid, either alone or along with a PFV Env expression vector. Virion and cell lysates were analyzed by Western blotting with αPFV human serum. (C) VLP production by 293T cells transfected with plasmids expressing PFV Gag appended with the indicated membrane-targeting signals.

Generation of PFV Gag virus-like particles in the absence of other virion proteins

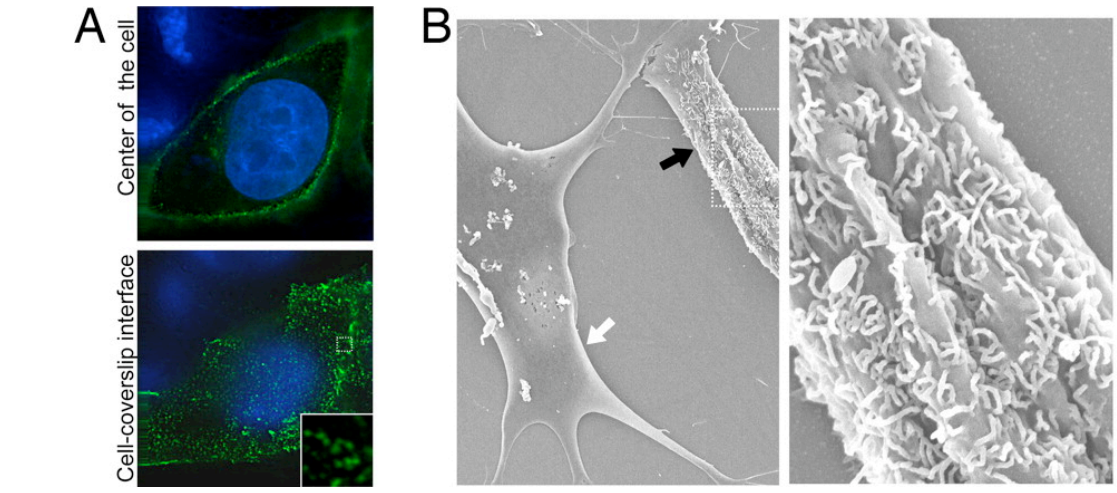
Previous work, using the Src oncoprotein myristoylation signal, has shown that appending the amino terminus of PFV Gag with a membrane-targeting signal can bypass the requirement for Env coexpression in particle release [251]. However, like the Src signal, most peripheral membrane-targeting signals contain lysine residues, complicating the interpretation of experiments that test the functional role of Gag ubiquitin acceptors. Therefore, we designed PFV Gag proteins appended with candidate lysine-free plasma membrane-targeting peptides. These consisted of the following: (i) an artificial membrane targeting peptide (MyrR), comprising the minimal six-residue myristoylation signal of HIV-1 Gag followed by an arginine-rich linker; (ii) a ten-amino-acid myristylated and palmitoylated peptide derived from the N-terminus of the Fyn oncoprotein, in which two lysines were mutated to arginine (FynR); and (iii) a naturally lysine-free ten-amino-acid myristoylated and palmitoylated peptide from the N-terminus of the Lck oncoprotein (Fig. 10A). Control experiments confirmed that the authentic PFV Gag protein, expressed from a PFV proviral plasmid, was released in extracellular particles only when Env was coexpressed (Fig. 10B). Concordantly, expression of the PFV Gag protein in the absence of any other viral protein did not generate VLPs (Fig. 10C). However, the membrane-targeted Gag proteins formed extracellular particles in the absence of Env expression. In the case of MyrR-Gag, VLP formation was inefficient, but the myristylated/palmitoylated Gag proteins (Fyn-Gag, FynR-Gag and Lck-Gag) efficiently generated extracellular VLPs (Fig. 10C). Since Lck-Gag formed VLPs with marginally greater efficiency than FynR-Gag, without introducing lysine residues, it was selected for further experiments.

Lck-Gag targets the plasma membrane and requires its PSAP motif to form extracellular VLPs

We next verified that our targeting strategy had the intended consequences. An Lck-Gag-CFP fusion protein efficiently targeted the plasma membrane in HeLa cells (Fig. 11A). Indeed, like most retroviral Gag-GFP fusion proteins, Lck-Gag-CFP was visible as fluorescent puncta at the cell surface, particularly when the cell surface-coverslip interface was imaged. To confirm that Lck-Gag assembled into VLPs, it was expressed in HeLa and DF1 cells, which have unusually smooth surfaces, facilitating the detection of surface VLPs using scanning electron microscopy. Although we sometimes observed short filamentous particles on the surface of Lck-Gag-expressing HeLa or DF1 cells, it was often difficult to observe retroviral VLPs by this technique, unless their release was arrested by coexpressing a dominant negative version of Vps4 [Vps4 (E228Q)]. We observed numerous short filamentous particles extending from the surface of DF1 cells coexpressing Lck-Gag and Vps4 (E228Q) (Fig. 11B). While these differed from the spherical cytoplasmic particles that were assembled by non-membrane targeted PFV Gag, they were indistinguishable from those assembled by MLV Gag, which have been shown to be filamentous in the absence of co-expressed glycosylated Gag [254] or when L-domain function was inhibited [110,115].

As additional verification of the authenticity of Lck-Gag assembly, we determined whether VLP release required an intact L-domain. Indeed, both the membrane targeting signal and the PSAP motif in Lck-Gag were required for VLP release (Fig. 11C). Thus, Lck-Gag mimics a conventional retroviral Gag protein in that it

assembles and is released at the cell surface via a plasma membrane-targeting signal and late domain-dependent engagement of the class E VPS machinery.



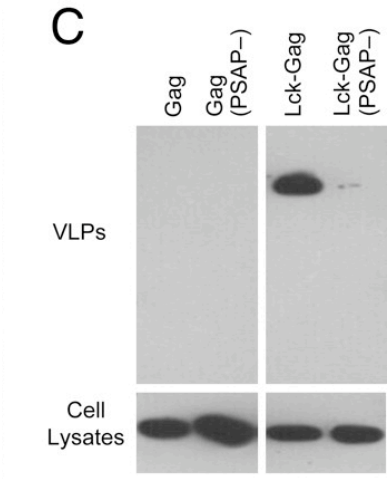


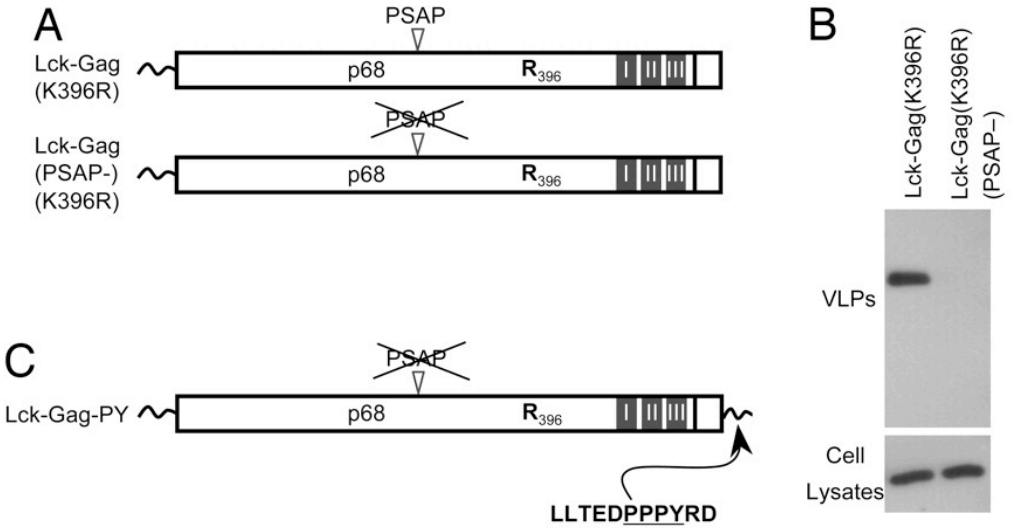
Figure 11. Lck-Gag forms VLPs at the plasma membrane that are released in a late domain-dependent manner. (A) HeLa cells expressing Lck-Gag-CFP (green) were fixed, and nuclei were stained with Hoechst (blue). Single deconvolved optical sections acquired at the center of the vertical dimension of the cell (Upper) or at the cell-coverslip interface (Lower) are shown. (B) Scanning electron micrograph of DF1 cells transfected with Lck-Gag and GFP-VPS4(E228Q). Representative transfected (black arrow) and untransfected (white arrow) cells, identified by GFP expression, are shown (Left). A higher-magnification view of area indicated by the dashed box is also shown (Right). (C) VLP production by 293T cells expressing wild-type PFV Gag or Lck-Gag proteins encoding either wild-type or mutant ($PSAP_{284–287}$ mutated to AAAT) late domains.

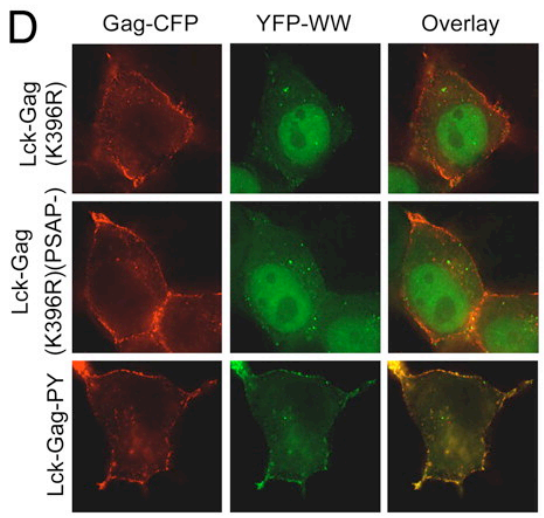
Class E VPS machinery- and ubiquitin ligase-dependent budding of lysine-free Gag

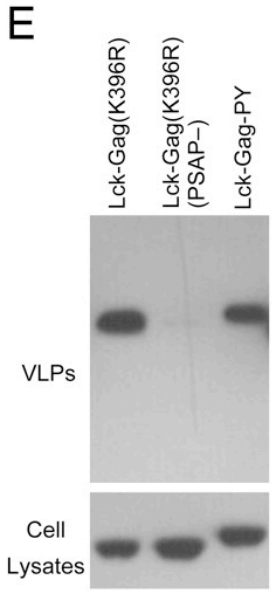
Next, we constructed Gag proteins devoid of ubiquitin acceptors by replacing the single lysine in Lck-Gag and Lck-Gag(PSAP-) with arginine (Fig. 12A). Because the amino terminus of Lck-Gag is occupied by myristoylation, this manipulation should remove all potential ubiquitin acceptor sites from Lck-Gag. Remarkably, the Lck-Gag(K396R) protein efficiently generated extracellular VLPs (Fig. 12B), and this was dependent on engagement of the class E VPS machinery, because Lck-Gag(K396R)(PSAP-) failed to generated extracellular VLPs (Fig. 12B). Notably, the efficiency with which Lck-Gag and Lck-Gag(K396R) formed VLPs was indistinguishable, strongly suggesting that viral protein ubiquitination is dispensable for the function of PT/SAP late domains.

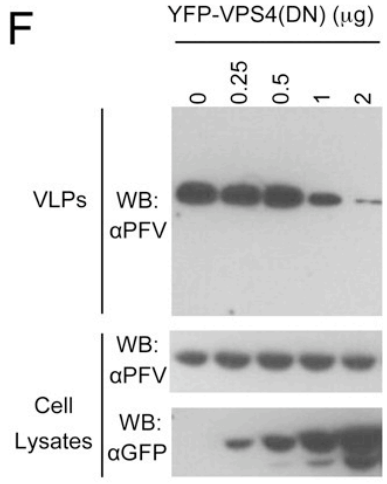
Because P(T/S)AP-type late domains directly bind to Tsg101 to recruit the class E VPS machinery, they could conceivably bypass the requirement for ubiquitination and thereby supplant the perceived role of ubiquitin in viral budding. Therefore, we next determined whether Gag ubiquitination is required for the function of a late domain that acts via ubiquitin ligase recruitment. To this end, we appended a murine leukemia virus (MLV)-derived late domain to the C-terminus of Lck-Gag(K369R) in which the PSAP motif had been inactivated, generating Lck-Gag-PY (Fig. 12C). PPxY motifs recruit HECT ubiquitin ligases by binding their WW domains; therefore, to verify that the transplanted MLV-derived late domain could indeed recruit a ubiquitin ligase that can induce MLV budding (WWP1) [115], Lck-Gag-PY-CFP was coexpressed with YFP fused to the WW domains of WWP1 (YFP-WW). The YFP-WW fusion protein

Figure 12. PT/SAP and PPxY-type late domains induce budding of lysine-free Gag proteins. (A) Schematic representation of lysine-free Lck-Gag proteins. (B) Western blot (αPFV) analysis of Lck-Gag expression in and VLP release from 293T cells transfected with Lck-Gag(K396R) expression plasmids encoding either the wild-type or mutant late domains. (C) Schematic representation of the Lck-Gag-PY protein, which is lysine free and encodes an MLV-derived late domain, appended at its C terminus. (D) YFP-WW fusion protein localization in HeLa cells coexpressing lysine-free Lck-Gag-CFP proteins encoding a PSAP motif, no late domain, or the MLV late domain, as indicated. (E) Western blot analysis (αPFV) of VLP production by 293T cells expressing lysine-free Lck-Gag proteins encoding the indicated late domains. (F) Western blot analysis (αPFV) (Top and Middle) of VLP production by 293T cells expressing Lck-Gag-PY. Cells were cotransfected with the indicated amounts of a YFP-VPS4(E228Q) plasmid, the expression of which was monitored by using αGFP Western blotting (Bottom).


A
PSAP
Lck-Gag
(K396R)
p68
R396
PSAP
Lck-Gag
(PSAP-)
(K396R)
p68
R396
B
Lck-Gag(K396R)
Lck-Gag(K396R)
(PSAP–)
VLPs
Cell
Lysates
C
PSAP
Lck-Gag-PY
p68
R396
LLTEDPPPYRD


D
Gag-CFP
YFP-WW
Overlay
Lck-Gag
(K396R)
Lck-Gag
(K396R)(PSAP-)
Lck-Gag-PY


E
Lck-Gag(K396R)
Lck-Gag(K396R)
(PSAP–)
Lck-Gag-PY
VLPs
Cell
Lysates


F
YFP-VPS4(DN) (μg)
0
0.25
0.5
1
2
VLPs
WB:
αPFV
WB:
αPFV
Cell
Lysates
WB:
αGFP

constitutively localizes to the nucleus but is relocalized, in a PPxY dependent manner, when coexpressed with MLV Gag or Ebola virus matrix [115]. Notably, YFP-WW was efficiently recruited to the plasma membrane in cells coexpressing Lck-Gag-PY-CFP, but remained in the nucleus of cells expressing Lck-Gag proteins lacking the MLV late domain (Fig. 12D). Thus, the MLV PPxY motif was capable of recruiting WWP1 in the context of Lck-Gag-PY.

Remarkably, The PPxY late domain stimulated the budding of lysine-free Lck-Gag (Fig. 12E), albeit less efficiently than the natural PSAP late domain (Fig. 12E). Moreover, Lck-Gag-PY particle release was inhibited by Vps4 (E228Q) overexpression, indicating that the requirement for a functional class E VPS pathway in PPxY-dependent budding of PFV Gag was maintained (Fig. 12F). Thus, a late domain that functions by recruiting ubiquitin ligases appeared fully capable of inducing the release of VLPs using the Class E VPS machinery, despite the absence of ubiquitin acceptor sites in the viral protein.

A catalytically active ubiquitin ligase (WWP1) can stimulate budding of a viral protein lacking ubiquitin acceptors

PPxY-dependent virus budding can sometimes be stimulated by overexpression of a HECT ubiquitin ligase. This effect is particularly evident with an attenuated MLV PPxY mutant exhibiting a partial budding defect that can be rescued by WWP1 overexpression [115]. Notably, overexpression of YFP-WWP1 markedly enhanced the release of particles generated by Lck-Gag-PY (Fig. 13A). This effect required the PPxY motif in Lck-Gag-PY, because PSAP-dependent Lck-Gag VLP release was not enhanced

(Fig. 13B), and release of an Lck-Gag lacking a functional late domain was not induced by WWP1 overexpression (Fig. 13C). Despite the fact that Lck-Gag-PY lacked ubiquitin acceptor sites, stimulation of VLP release required WWP1 catalytic activity. Indeed, expression of a truncated WWP1 lacking the catalytic domain (WWP1-ΔHECT) or an enzymatically inactive point mutant [WWP1(C890S)] inhibited rather than stimulated Lck-Gag-PY release (Fig. 13A). Again, these effects were specific to the PPxY late domain, as PSAP-dependent and late domain-independent Lck-Gag VLP release was not affected (Fig. 13B). Thus, Lck-Gag-PY can recruit WWP1, but the effect of this recruitment on particle budding was critically dependent on the catalytic activity of WWP1, even in the complete absence ubiquitin acceptor sites in the viral protein.

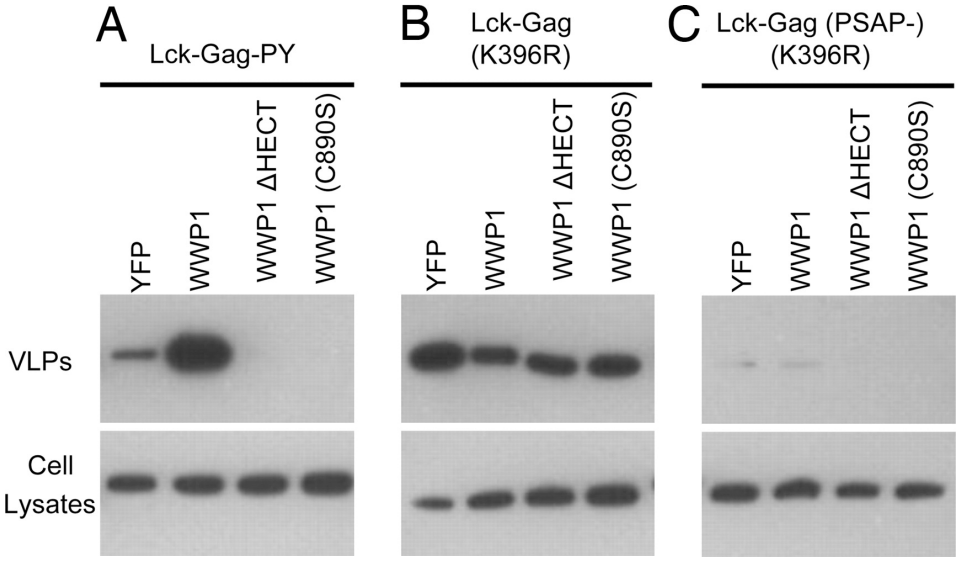


Figure 13. Ubiquitin-ligase-stimulated, PPxY-dependent budding of lysine-free Gag. Western blot (αPFV) analysis of Gag expression and VLP production by 293T cells expressing lysine-free Lck-Gag proteins: Lck-Gag-PY (A); Lck-Gag(K396R) (B); or Lck-Gag(PSAP-)(K396R) (C). Each Lck-Gag protein was coexpressed with unfused YFP as a control or YFP fused to WWP1, WWP1 lacking the HECT domain (WWP1-ΔHECT), or a catalytically inactive mutant of WWP1 [WWP1(C890S)], as indicated.

Effects of addition of ubiquitin acceptor sites on Gag ubiqitination

To exclude the possibility that our PFV Gag proteins became ubiquitinated by an alternative mechanism, independent of lysine or amino terminal acceptors, we determined whether ubiquitinated Gag proteins were generated in our assays. As a positive control, we prepared an Lck-Gag expression plasmid containing late domain-proximal ubiquitin acceptors by appending three lysine residues to the C-terminus of Lck-Gag-PY (generating Lck-Gag-PY-3K, Fig. 14A). We immunoprecipitated Lck-Gag proteins from 293T cell lysates, prepared twenty-four hours after co-transfection with plasmids expressing (i) Lck-Gag variants, (ii) HA-tagged ubiquitin, and (iii) wild-type or inactive mutant WWP1. Thereafter, ubiquitinated Lck-Gag species were detected by immunoblotting. Lck-Gag proteins were efficiently immunoprecipitated and detected using αPFV antibody (Fig. 14B), but ubiquitinated Lck-Gag was undetectable in cells expressing lysine-free proteins, regardless of which late domain was present, and even when WWP1 was overexpressed (Fig. 14B). However, immunoprecipitates from cells expressing Lck-Gag-PY-3K contained several HA-reactive species corresponding to mono and oligoubiquitinated Gag (Fig. 15B upper panel). A secondary band of slightly higher molecular weight than Lck-Gag-PY-3K, likely corresponding to a monoubiquitinated species, was also detected using αPFV in cells expressing Lck-Gag-PY-3K, but not Lck-Gag-PY (Fig. 15B, center and lower panels). These ubiquitinated forms reflect HECT ligase-mediated ubiquitination, as they were observed only in cells overexpressing catalytically active, but not catalytically inactive, WWP1. Thus, despite the ability of Lck-Gag-PY to recruit a ubiquitin ligase, its ubiquitination was detected

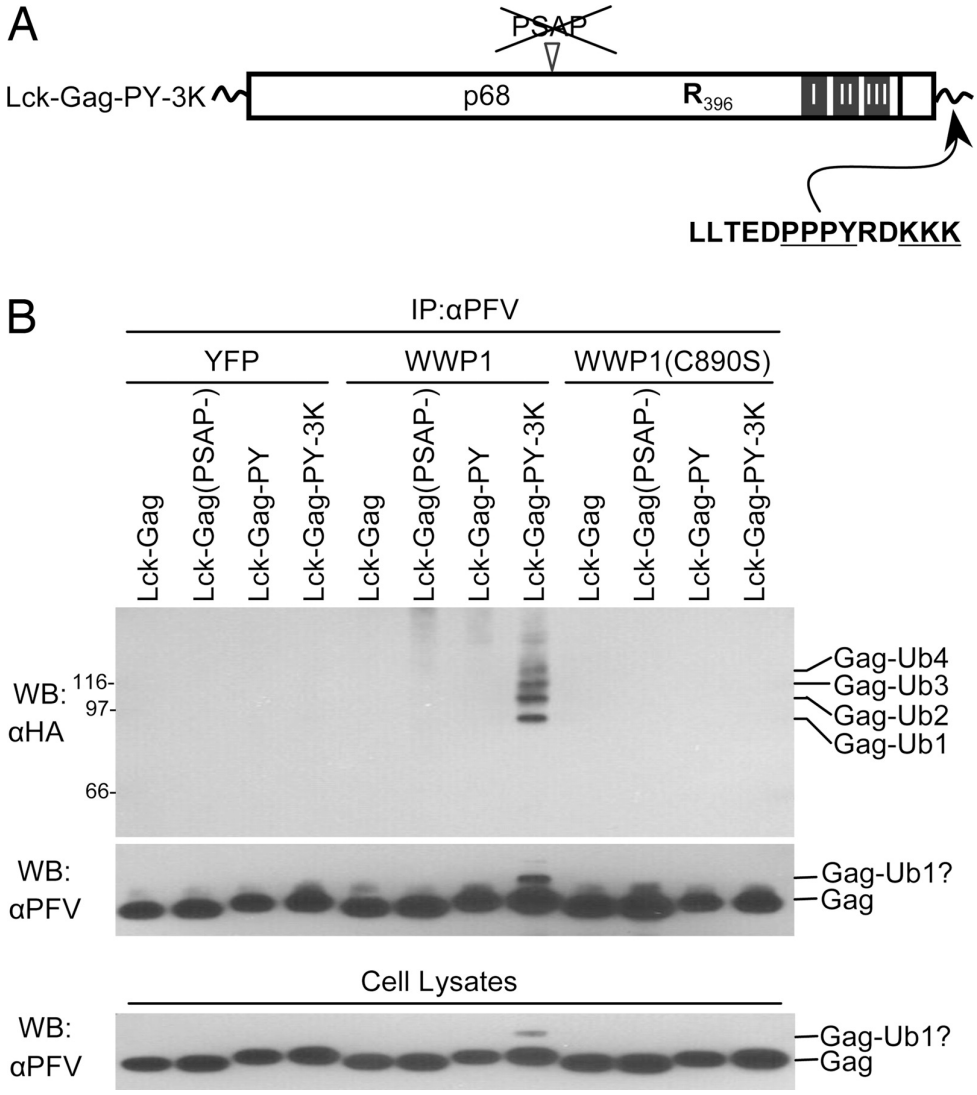


Figure 14. Lysine-dependent, WWP1-induced ubiquitination of Lck-Gag. (A) Schematic representation of the Lck-Gag protein appended with the MLV late domain and three lysine residues at its C terminus (Lck-Gag-PY-3K). (B) Western blot (WB)/immunoprecipitation (IP) analysis of Lck-Gag proteins encoding the indicated late domains from 293T cells cotransfected with plasmids expressing Lck-Gag proteins, HA-ubiquitin, and unfused YFP, YFP-WWP1, or YFP-WWP1(C890S). All Lck-Gag proteins were lysine free except Lck-Gag-PY-3K. (Top and Middle) The αPFV immunoprecipitates were analyzed by Western blotting with an αHA monoclonal antibody (Top) or αPFV serum (Middle). (Bottom) Alternatively, unfractionated cell lysates were probed with αPFV serum. For the αHA blot, the migration of molecular weight markers is indicated to facilitate assignment of ubiquitinated forms.

only as a consequence of insertion of lysine residues at the C-terminus. Therefore, lysine-free Lck-Gag proteins appear to be resistant to ubiquitination by WWP1.

Lck-Gag ubiquitination does not enhance particle release

We next determined whether the presence of ubiquitin acceptors, and ubiquitin conjugation, could enhance PPxY-dependent particle release. First, we compared Lck-Gag-PY with Lck-Gag-PY-3K, whose release is dependent on HECT ubiquitin ligases and differ only in the presence or absence of late domain-proximal ubiquitin acceptors. Notably, Lck-Gag-PY-3K generated extracellular VLPs with no greater efficiency than lysine-free Lck-Gag-PY (Fig. 15A, left panel). Moreover, ubiquitinated Gag species were detectable in VLPs assembled by Lck-Gag-PY-3K, but not lysine free Gag proteins (Fig. 15A, right panel). Thus, under conditions where ubiquitinated Gag is, or is not, generated, VLP release was equivalent. Additionally, WWP1 stimulated the release of particles generated by using Lck-Gag-PY-3K (Fig. 15B, C). Importantly, the magnitude of this effect was no greater than that observed with lysine-free Lck-Gag (Fig. 15B). Thus, WWP1-stimulated, PPxY-dependent particle release occurred equivalently whether or not it was accompanied by Gag ubiquitination.

Summary

We generated a plasma membrane-targeted lysine-free PFV Gag protein (Lck-PFV Gag) that can assemble at the cell surface and form extracellular virus-like particles in the absence of other viral protein expression. Notably, direct Gag ubiquitination was

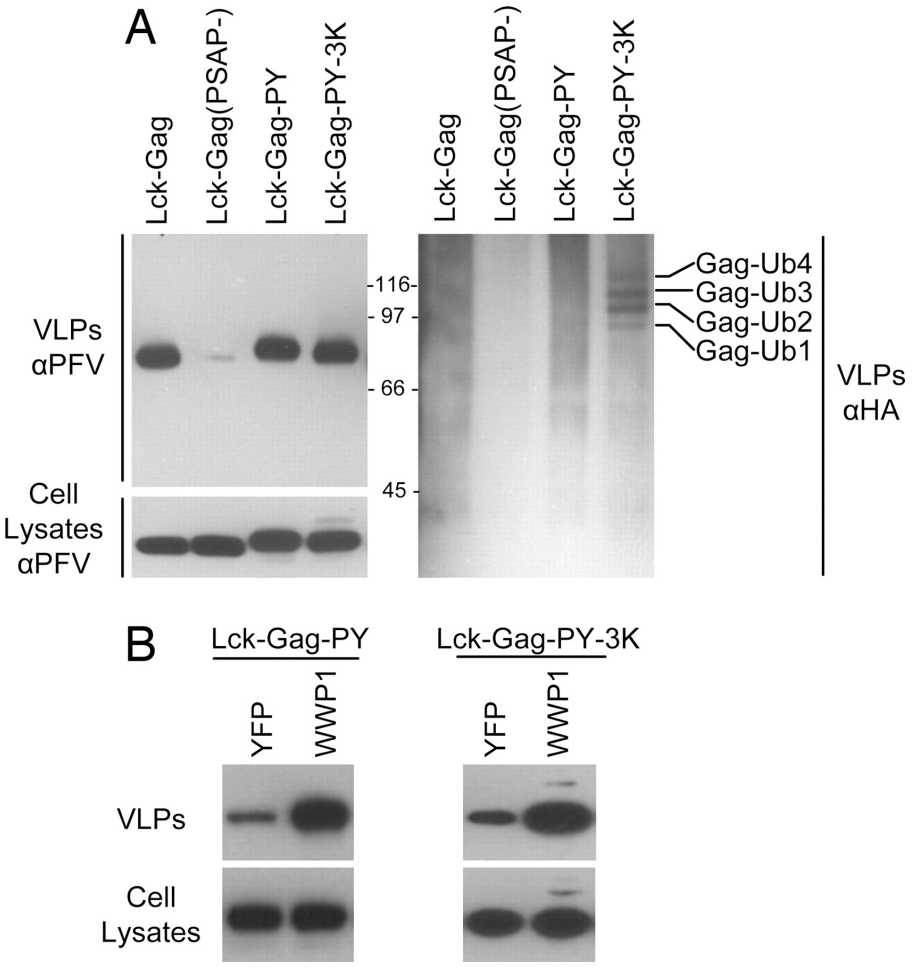


Figure 15. Presence of ubiquitin acceptors or Gag ubiquitination does not affect ubiquitin-ligase-induced Lck-Gag budding. (A) Western blot analysis of VLP production by 293T cells coexpressing HA-ubiquitin and the indicated PFV Gag proteins. VLP lysates were analyzed by Western blotting with αPFV serum (Left) or monoclonal αHA antibody (Right). Migration of molecular weight markers is indicated to facilitate assignment of ubiquitinated forms. (B) Western blot (αPFV) analysis of VLP production by Lck-Gag-PY proteins that were lysine-free or that bore three appended lysine residues at the C- terminus, as indicated. The Lck-Gag-PY proteins were expressed either with unfused YFP, as a control, or with YFP-fused WWP1, as indicated.

dispensable for both PSAP- and PPxY-dependent budding of Lck-PFV Gag. Moreover, overexpression of HECT ubiquitin ligase WWP1 specifically stimulated PPxY-dependent particle production by lysine-free Lck-Gag. Importantly, the catalytic activity of WWP1 was required to promote PPxY-dependent budding in the presence and absence of ubiquitin acceptor sites within Gag. Taken together, our findings suggest that HECT ligases mediate late domain dependent budding by ubiquitinating transacting cellular factors and that ubiquitination of Gag is merely a side effect of ubiquitin ligase recruitment.

Chapter IV. Comparative analysis of HECT domain function

The Class E VPS pathway must be intact to support HECT ubiquitin ligase-dependent virion release. The yeast HECT ligase Rsp5 is dispensable for MVB biogenesis but required for sorting of some endosomal cargoes [225]. In essence, PPxY late domains allow viral Gag proteins to be recognized by the Class E pathway as a specific type of cargo by recruiting HECT ligases, but the biochemical link between the ligases and the VPS machinery remains elusive.

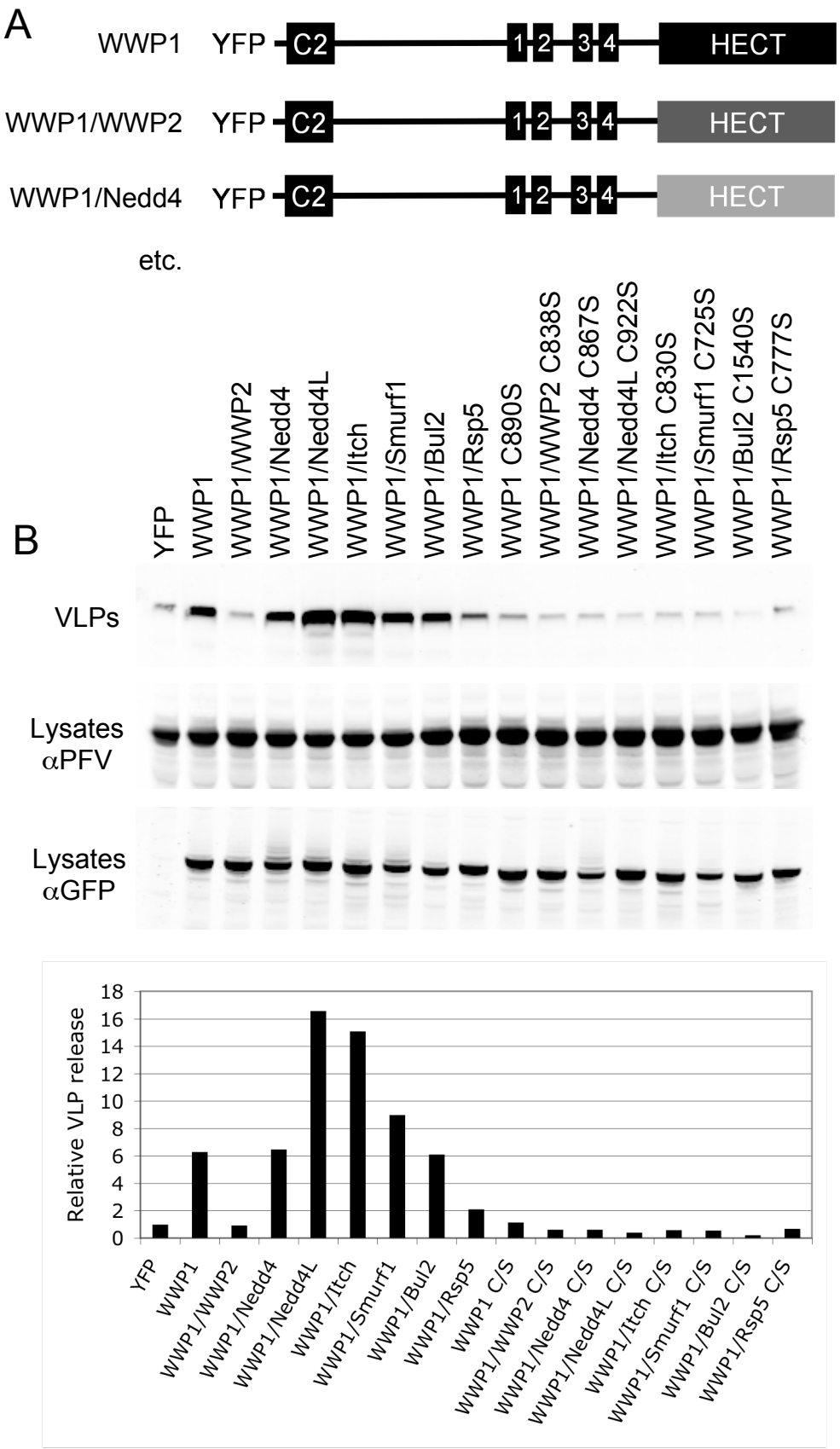
A wild-type catalytic domain is essential for HECT ligase-mediated enhancement of PPxY-dependent budding [115,255], but the precise mechanism of this stimulation is unclear. Expression of enzymatically inactive WWP1, containing a single point mutation that disrupts its active site (C890S), fails to stimulate PPxY-dependent budding ([115] and Fig. 14B). Moreover, expression of truncated WWP1, lacking the entire catalytic domain, induces a more potent dominant negative effect on MLV budding than overexpression of the catalytic point mutant [115], suggesting that the HECT domain may encode additional elements that contribute to its ability to engage the Class E VPS machinery. Indeed, WWP1 localizes to aberrant endosomal compartments induced by overexpression of dominant negative Vps4, and this recruitment is largely dependent on its HECT domain [115]. Thus, HECT domains may mediate the interaction of full-length ubiquitin ligases with VPS factors, which are trapped on these aberrant compartments. Such interactions have not been convincingly documented by protein-protein binding assays and may be indirect, requiring unidentified bridging factors.

Nine members of the Nedd4-like HECT ubiquitin ligase family have been described in humans, with regulatory roles in cell signaling, endocytosis, protein trafficking, and viral budding (reviewed in [209]). These ubiquitin ligases vary in their ability to promote PPxY-dependent MLV virion release, due at least in part to differences in the abilities of their WW domains to bind the MLV late domain [115]. A comparative analysis of HECT domain activities, including substrate ubiquitination and budding stimulation, could provide useful clues about their mechanistic role in VPS pathway engagement.

Catalytically active HECT domains stimulate PPxY-dependent budding with variable efficiency

To distinguish the features of HECT domains that are important for stimulation of virus particle release we compared the various activities of isolated HECT domains. Since WWP1 has been previously shown to strongly interact with the MLV late domain [115], we constructed a series of chimeric ubiquitin ligases, consisting of the membrane targeting and PPxY motif-binding domains (C2-WW) of human WWP1 and different catalytic domains, derived from human WWP2, Nedd4, Nedd4L, Itch, Smurf1, or Bul2 or yeast Rsp5 HECT ligases (Fig. 16A). Overexpression of chimeric HECT ligases markedly reduced the expression of MLV GagPol in cell lysates (data not shown), perhaps by inducing its degradation, making it difficult to compare their effects on budding. Thus, we used a Gag protein containing minimal ubiquitin acceptor lysine residues. Lysine-free Prototype foamy virus (PFV) Gag (Lck-PFV Gag) can assemble at the plasma membrane and bud in the absence of other viral protein expression, using both

Figure 16. Stimulation of PPxY-dependent VLP production by chimeric HECT ligases. (A) Schematic representation of YFP-fused chimeric ubiquitin ligases containing the membrane targeting (C2) and late domain binding (WW) domains of WWP1 and the indicated catalytic HECT domains. Wild-type and catalytically inactive mutant (C-S) versions of each HECT ligase were constructed. (B) Quantitative Western blot (LICOR) analysis of VLP release from 293T cells coexpressing Lck-Gag-PY and the indicated chimeric HECT ligases.


A
WWP1
YFP
C2
1
2
3
4
HECT
WWP1/WWP2
WWP1/Nedd4
etc.
B
YFP
WWP1
WWP1/WWP2
WWP1/Nedd4
WWP1/Nedd4L
WWP1/Itch
WWP1/Smurf1
WWP1/Bul2
WWP1/Rsp5
WWP1 C890S
WWP1/WWP2 C838S
WWP1/Nedd4 C867S
WWP1/Nedd4L C922S
WWP1/Itch C830S
WWP1/Smurf1 C725S
WWP1/Bul2 C1540S
WWP1/Rsp5 C777S
VLPs
Lysates αPFV
Lysates αGFP
Relative VLP release
0
2
4
6
8
10
12
14
16
18
YFP
WWP1
WWP1/WWP2
WWP1/Nedd4
WWP1/Nedd4L
WWP1/Itch
WWP1/Smurf1
WWP1/Bul2
WWP1/Rsp5
WWP1 C/S
WWP1/WWP2 C/S
WWP1/Nedd4 C/S
WWP1/Nedd4L C/S
WWP1/Itch C/S
WWP1/Smurf1 C/S
WWP1/Bul2 C/S
WWP1/Rsp5 C/S

its naturally encoded PSAP late domain or a heterologous PPxY late domain, derived from MLV Gag. Expression of ubiquitin ligases containing any wild-type HECT domain, except that of human WWP2 and yeast Rsp5, stimulated PPxY-dependent budding of lysine-free Lck-PFV Gag (Fig. 16B). The strongest stimulation was observed with Nedd4L and Itch HECT domains. In contrast, overexpression of all enzymatically inactive chimeric ligases, in which the catalytic cysteine was mutated to serine, inhibited PPxY-dependent VLP production, suggesting that the catalytic activity of HECT domains is indispensable for stimulation of virion release.

Stimulation of PSAP-dependent VLP release by recruitment of catalytically active HECT domains

The VPS factor Tsg101 functions essentially as an adaptor protein, which recruits ESCRT-I proteins to Gag. The N-terminal ubiquitin E2 variant (UEV) domain of Tsg101 interacts with ubiquitin and P(T/S)AP peptide motifs [103,147,148,256], whereas the C-terminal portion of the protein interacts with ESCRT-I components Vps28 and Vps37 [149,241,257]. To determine whether P(T/S)AP-dependent VLP production can be redirected through the ubiquitin ligase-dependent pathway, we constructed chimeric proteins containing the UEV domain (residues 1-157) of Tsg101 fused to one of several human HECT domains (Fig. 17A). To assess the enhancement of PSAP-dependent budding, we constructed an attenuated "leaky" budding mutant of Lck-PFV Gag by changing the first proline residue of the PSAP motif to alanine. Expression of chimeric Tsg-HECT proteins containing wild-type WWP1 and Nedd4L HECT domains induced partial rescue of Lck-Gag-ASAP budding, while expression of a chimeric protein

containing the Itch catalytic domain completely rescued particle production. Overexpression of catalytically inactive chimeric Tsg-HECT proteins inhibited Lck-PFV Gag-ASAP VLP release (Fig. 17B). Thus, ubiquitin ligase activity at sites of VLP assembly appears to stimulate particle production, irrespective of its mode of recruitment to Gag.

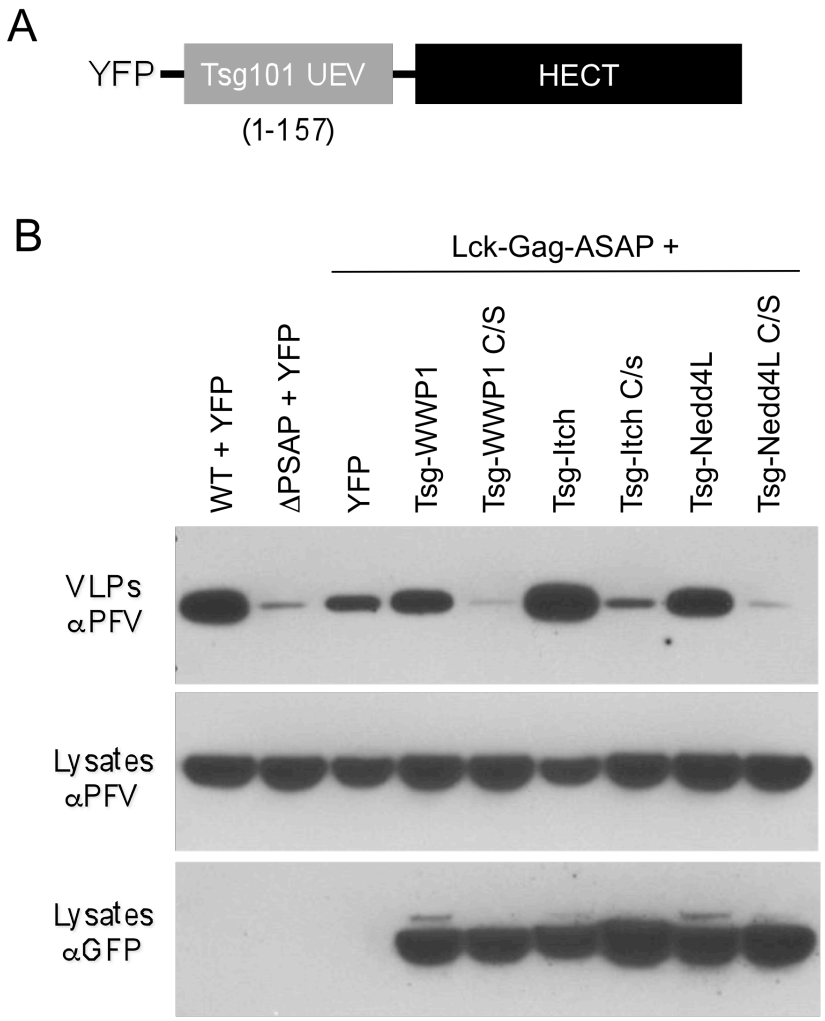


Figure 17. Stimulation of PSAP-dependent budding by catalytically active HECT domains. (A) Schematic representation of a chimeric ubiquitin ligase containing the UEV domain of Tsg101 (PSAP binding) and the catalytic domain of a HECT ligase. (B) VLP production from 293T cells expressing Lck-PFV Gag containing either a wild-type (PSAP), inactive (ΔPSAP), or attenuated (ASAP) late domain and the indicated chimeric ubiquitin ligase.

Stimulation of PFV Gag budding correlates with HECT ligase auto-ubiquitination rather than Gag ubiquitination

To assess the relative catalytic activities of HECT domains, we compared the abilities of chimeric HECT ligases to ubiquitinate themselves and a PFV Gag substrate, encoding three lysine residues in close proximity to a PPxY late domain (Lck-Gag-PY-3K). We immunoprecipitated PFV Gag and HECT ligases from 293T cell lysates, prepared 36 hours after co-transfection with plasmids expressing Lck-Gag-PY-3K, HA-tagged ubiquitin, and YFP-fused chimeric HECT ligases encoding wild-type catalytic domains. Cell lysates were prepared using detergent-rich buffer (0.5% SDS) to ensure complete dissolution of protein complexes, and ubiquitinated species were detected by immunoblotting with an αHA antibody.

All of the tested chimeric ligases induced PPxY-dependent PFV Gag ubiquitination (Fig. 18A), but no correlation between direct Gag ubiquitination and stimulation of VLP production was apparent. Bul2 and Nedd4 HECT domains, which induced the highest and lowest levels of Gag ubiquitination, respectively, stimulated budding to a similar extent (about 6-fold). Moreover, the Rsp5 HECT domain, which induced high levels of Gag ubiquitination, enhanced budding only marginally.

In contrast, we observed a broad, albeit imperfect correlation between the ability of HECT ligases to self-ubiquitinate and to stimulate VLP production (Fig. 18B). Ligases that strongly promoted particle release (Itch and Nedd4L HECT domains) carried more ubiquitin, while those that failed to promote VLP release (WWP2 and Rsp5 HECT domains) carried relatively little. The correlation was imperfect, however, since Nedd4, which moderately enhanced particle release, was consistently highly ubiquitinated.

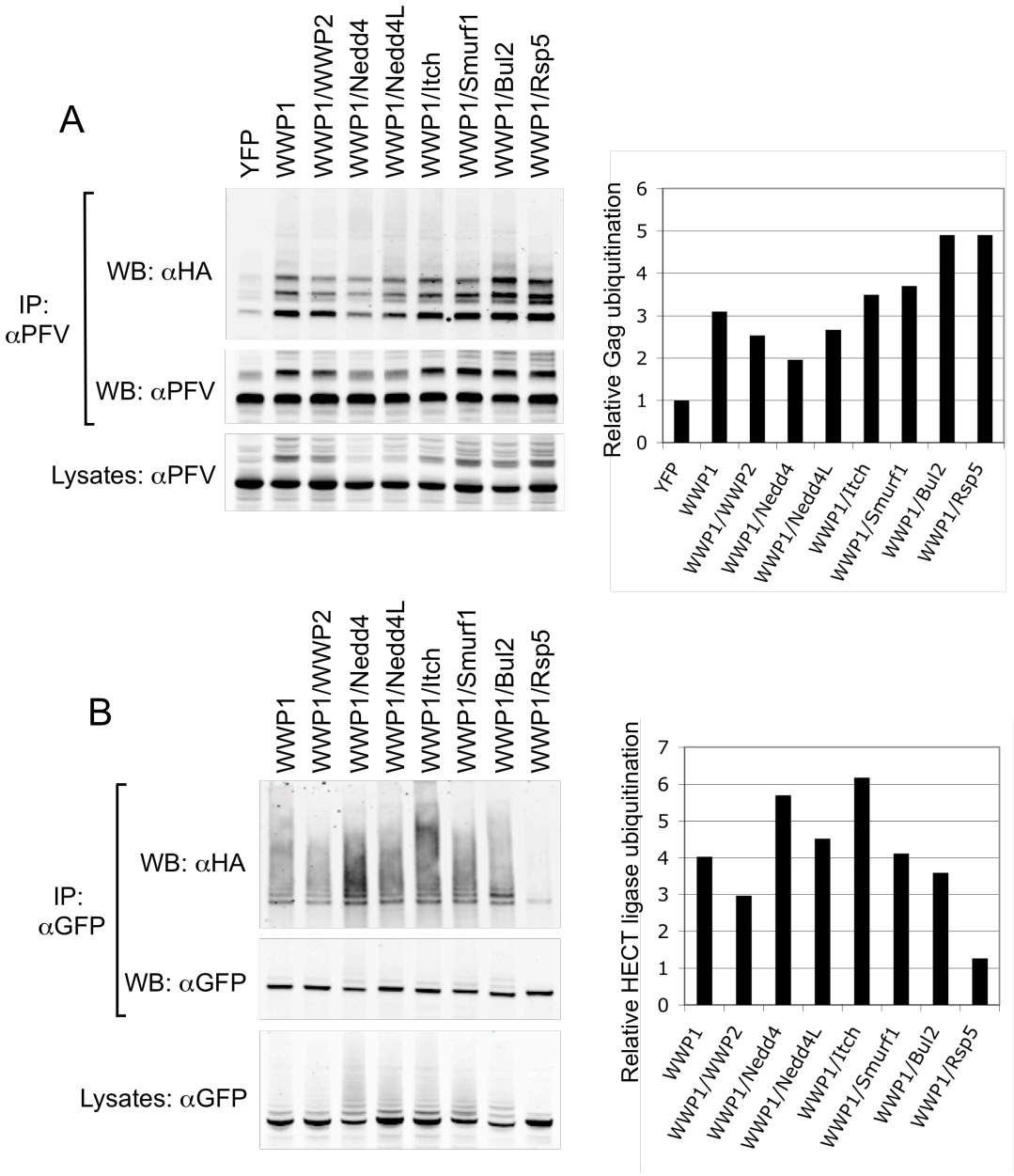


Figure 18. Chimeric HECT ligases induce different levels of Gag- and auto-ubiquitination. Quantitative Western blot analysis of lysine-containing PFV Gag proteins (A) and chimeric HECT ligase proteins (B), immunoprecipitated from 293T cells co-transfected with plasmids expressing Lck-Gag-PY-3K, HA-ubiquitin, and the indicated chimeric YFP-HECT ligases. The αPFV immunoprecipitates were probed with an αHA monoclonal antibody (A, top) or αPFV serum (A, middle). The αGFP immunoprecipitates were probed with αHA (B, top) and αGFP (B, middle) monoclonal antibodies. Alternatively, unfractionated cell lysates were probed with αPFV serum or αGFP antibody (A and B, bottom).

Notably, all HECT domains localized to Vps4-induced Class E compartments, but there was no apparent correlation between their recruitment to class E compartments and ability to stimulate budding (Fig. 19).

Our data support our previous finding that direct Gag ubiquitination is dispensable for HECT ligase-dependent budding [255] and suggest a hypothesis that the total amount of ubiquitin deposited at sites of virion assembly is important for stimulation of particle release, rather that the identity of the protein to which it is ligated. HECT ligase auto-ubiquitination might account for the majority of total ubiquitin present in the vicinity of PPxY-dependent Gag assembly. However, we have been unable to directly assess the total levels of protein ubiquitination induced by each chimeric ligase when it is in a complex with Gag, due to the inefficient solubility of the ubiquitinated complexes under conditions that preserve protein-protein interactions. Thus, we cannot rule out the possibility that HECT domains recruit VPS proteins, either directly or through bridging factors, since auto-ubiquitination of HECT ligases may enhance their affinity for downstream binding partners that, in turn, could allow Class E pathway engagement.

Stimulation of PPxY-dependent budding by direct recruitment of ESCRT-I

To determine whether the requirement for enzymatically active ubiquitin ligase recruitment for PPxY-dependent budding could be bypassed, we constructed chimeric proteins by replacing the HECT domain of WWP1 with the C-terminal portion of Tsg101 (TsgΔUEV, residues 157-390, Fig. 20A). Overexpression of WWP1-TsgΔUEV stimulated Lck-PFV Gag-PY budding in a dose-dependent manner but had no effect on particle production by late domain-deficient PFV Gag (Fig. 20B, left and middle panels).

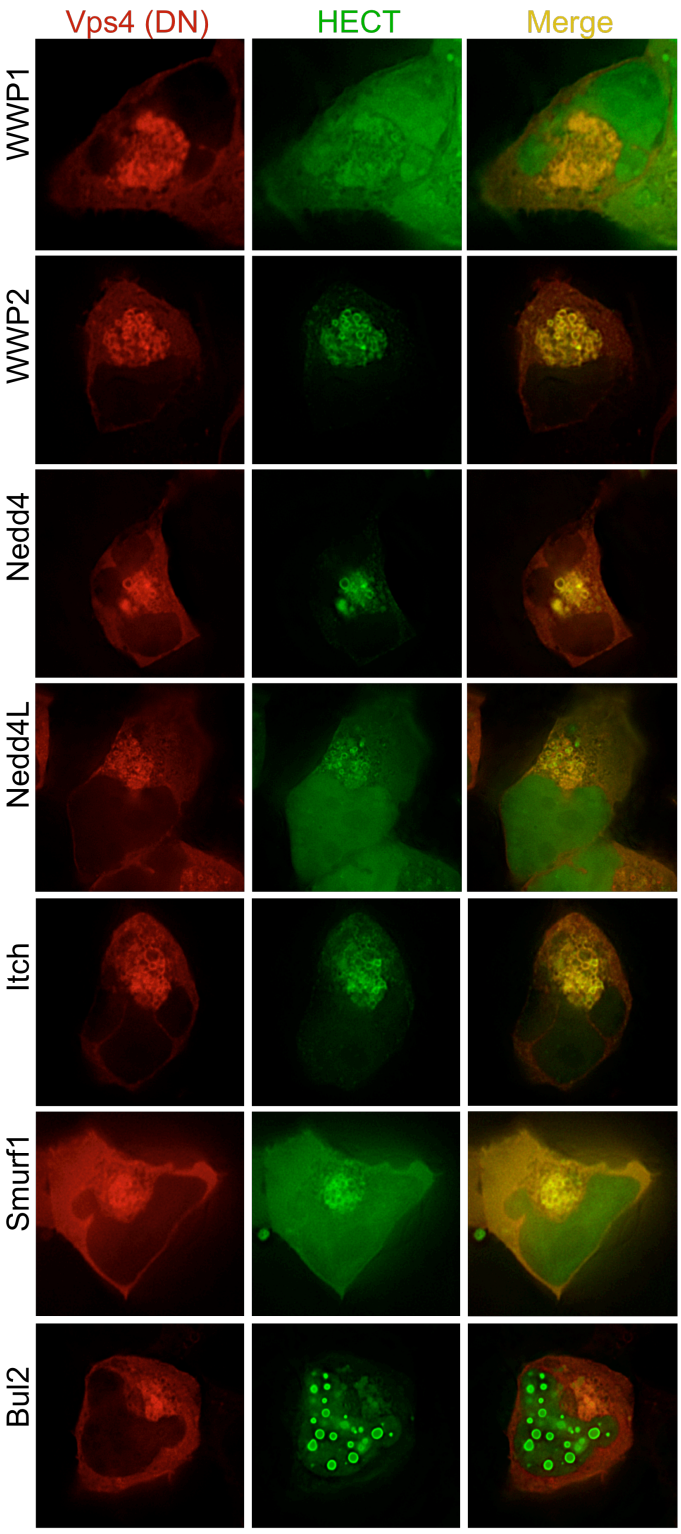


Figure 19. HECT domain recruitment to dominant negative Vps4-induced Class E compartments. Subcellular localization of indicated YFP-HECT domains in 293T cells coexpressing CherryFP-Vps4(E228Q).

Thus, the chimeric protein seems capable of recruiting a functional ESCRT-I complex to assembling virions that encode PPxY late domains, thereby stimulating VLP production. Interestingly, WWP1-TsgΔUEV overexpression inhibited PSAP-dependent Lck-PFV Gag budding in a dose-dependent manner (Fig. 11B, right panel). Presumably, since the chimeric protein lacks the structural elements required for interaction with PSAP motifs, it can act as a dominant negative inhibitor of PSAP-dependent budding by sequestering endogenous ESCRT-I components into complexes that cannot bind to PTAP motifs.

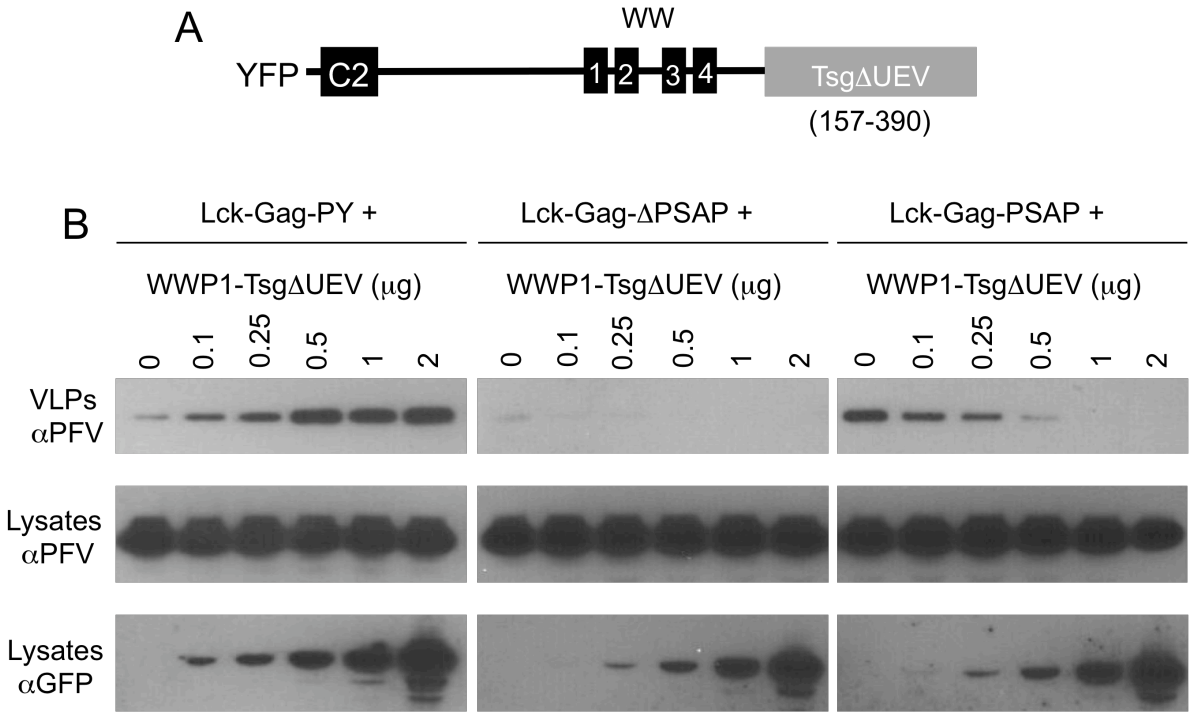


Figure 20. Stimulation of PPxY-dependent budding by direct recruitment of ESCRT-I. (A) Schematic representation of a chimeric protein designed to recruit ESCRT-I to PPxY late domains, containing the C2 and WW domains of WWP1 fused to a Tsg101 fragment lacking the UEV domain (TsgΔUEV). (B) VLP production from 293T cells expressing Lck-PFV Gag containing the indicated late domain and increasing amounts of YFP-WWP1-TsgΔUEV.

Summary

Humans encode several Nedd4-like ubiquitin ligases, which vary in their ability to stimulate PPxY-dependent MLV budding. HECT ligases may promote budding either by recruiting cellular factors to sites of virus particle assembly or by depositing ubiquitin onto proteins in the vicinity. To distinguish between these two alternatives we compared the capacity of different catalytic HECT domains to stimulate PFV budding and ubiquitination. We generated chimeric ubiquitin ligases that interact with either PPxY or PT/SAP-type late domains and contain different HECT domains. The ability of these ligases to stimulate PFV particle production was absolutely dependent on their catalytic activity, irrespective of their mode of recruitment to Gag. Moreover, the efficiency with which they stimulated budding correlated with the extent of their auto-ubiquitination. Interestingly, PPxY dependent budding could be enhanced in the absence of ubiquitin ligase activity by expression of a chimeric protein that directly recruits the ESCRT-I complex.

Chapter V. Direct ubiquitin fusion to Gag replaces late domain function

Stimulation of PPxY-dependent viral budding is strictly dependent on the catalytic activity of HECT ubiquitin ligases. However, the strength of the stimulation correlates, broadly, with the extent of HECT ligase autoubiquitination rather than Gag ubiquitination. This is consistent with several possible models for HECT ligase-mediated budding. HECT domains may contain structural elements that are normally inaccessible but can be activated to engage the Class E VPS pathway by conformational changes, induced by ubiquitination. Alternatively, HECT domains may contain suboptimal binding sites, whose affinity for class E factors or bridging factors is enhanced by ubiquitination. Finally, ubiquitin itself could serve as a docking site for Class E factors, many of which contain ubiquitin interacting motifs, and the actual identity of the substrate for ubiquitination may be unimportant, provided that it is proximal to the site of particle budding. In this case, HECT ligase autoubiquitination could simply represent the majority of ubiquitin present at viral assembly sites, since ubiquitin acceptor lysine residues are much more abundant in the ligases than in PFV Gag.

If the substrate for HECT ligase-mediated ubiquitination is indeed irrelevant, and PPxY late domains merely serve to concentrate ubiquitinated protein species in the vicinity of virus particle assembly, direct fusion of ubiquitin to Gag may obviate the need for a conventional late domain. Two previous studies have described late domain-like activity of ubiquitin that has been appended directly to Gag. Ubiquitin fusion to the C-terminus of RSV Gag rescues virus-like particle production in cells treated with

proteasome inhibitors but fails to compensate for the deletion of the PPxY late domain [104]. Fusion of ubiquitin to EIAV Gag promotes VLP release by late domain-deficient Gag but does not enhance budding of Gag encoding functional late domains [258]. Moreover, ubiquitin-dependent budding of EIAV Gag requires an intact Class E VPS pathway and a key hydrophobic surface patch of ubiquitin that mediates its recognition by ubiquitin-binding proteins. Lysine free PFV Gag, which cannot otherwise be modified by ubiquitin, offers a unique context for testing the effect of Gag-ubiquitin fusion on VLP production.

Direct fusion of ubiquitin to PFV Gag promotes VLP release in the presence and absence of conventional late domains

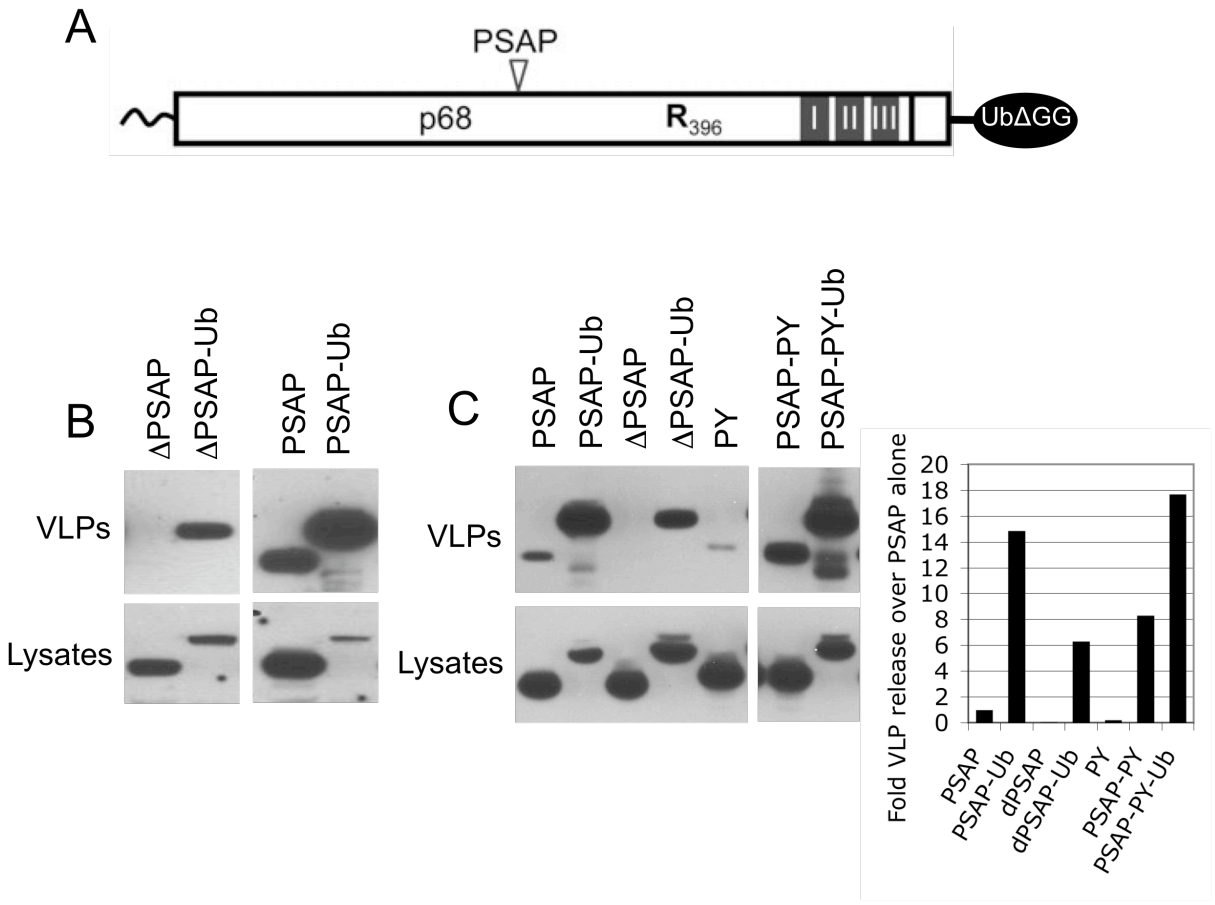
To mimic the deposition of ubiquitin at sites of virion assembly without HECT domain recruitment we generated membrane targeted PFV Gag constructs with a single ubiquitin appended at the C-terminus. Ubiquitin is conjugated to proteins by an isopeptide bond between the C-terminal glycine residue of ubiquitin and the ε-amino group of a lysine residue within the substrate protein. Thus, to avoid aberrant linkage of our Gag-ubiquitin chimeras to other proteins we deleted two glycine residues from the C-terminus of ubiquitin (Fig. 21A).

Remarkably, cells expressing ubiquitin-fused late domain-deficient Lck-PFV Gag (ΔPSAP-Ub) efficiently produced VLPs, whereas those expressing its unfused counterpart (ΔPSAP) produced undetectable levels (Fig. 21B, left panel). Moreover, attachment of ubiquitin to the C-terminus of PFV Gag containing a wild-type late domain (PSAP-Ub) greatly enhanced its particle production (Fig. 21B, right panel). To determine

the amount of ubiquitin necessary to promote PFV budding we titrated ubiquitin-fused Gag in a VLP production assay while keeping total levels of Gag constant. In the absence of conventional late domains, almost all of the Gag proteins must carry ubiquitin to support efficient VLP release (Fig. 21D, left panel). However, in the presence of a PSAP late domain, potent stimulation of VLP release was observed when only 25% of Gag carried ubiquitin (Fig. 21D, right panel). Thus, ubiquitinated species be present at sites of VLP assembly stimulate budding, in an apparently dose dependent manner. Moreover, ubiquitin appears to synergize with a late domain (i.e. a PTAP motif) to promote efficient VLP release.

Several enveloped viruses encode both PT/SAP and PPxY motifs within a single structural protein, with one typically playing a dominant role in promoting particle release. The activity of the secondary late domain usually becomes apparent only when the primary late domain is inactivated [193,259,260,261]. In the case of lysine-free Lck-PFV Gag, PSAP- and PPxY-dependent budding is relatively inefficient. However, when both late domains are present together within the same Gag protein, their activities appear to synergize, resulting in potent stimulation of VLP release (Fig. 21C). Direct fusion of ubiquitin to the C-terminus of Gag presumably mimics the effect of efficient recruitment of a highly active ubiquitin ligase by a PPxY-type late domain. Hence, ubiquitin fusion to PFV Gag induces greater stimulation of VLP release than addition of a PPxY late domain. Moreover, particle production can be further enhanced by fusing ubiquitin to PFV Gag that encodes both the PSAP and PPxY late domains (Fig. 21C).

Figure 21. Direct fusion of ubiquitin to Lck-PFV Gag stimulates late domain-dependent and -independent budding. (A) Schematic representation of lysine-free Lck-PFV Gag with a single ubiquitin moiety fused at the carboxy terminus. Two glycine residues were removed from the C-terminus of ubiquitin to prevent its conjugation to other proteins. (B) VLP production from 293T cells expressing the indicated Lck-PFV Gag constructs. (C) Relative VLP release efficiency by 293T cells expressing Lck-PFV Gag containing the indicated late domains, alone or in combination with ubiquitin fusion, measured by quantitative western blot. (D) VLP production from 293T cells expressing Lck-Gag and Lck-Gag-Ub containing either the wild-type (PSAP) or inactive (ΔPSAP) late domain. Cells were transfected with equal total amounts of Gag DNA, with the indicated fraction of Lck-Gag-Ub.


A
PSAP
p68
R396
UbΔGG
B
ΔPSAP
ΔPSAP-Ub
PSAP
PSAP-Ub
VLPs
Lysates
C
PSAP
PSAP-Ub
ΔPSAP
ΔPSAP-Ub
PY
PSAP-PY
PSAP-PY-Ub
VLPs
Lysates
Fold VLP release over PSAP alone
0
2
4
6
8
10
12
14
16
18
20
PSAP
PSAP-Ub
dPSAP
dPSAP-Ub
PY
PSAP-PY
PSAP-PY-Ub
D
Lck-PFV Gag-ΔPSAP
Fraction Gag-Ub
Lck-PFV Gag-PSAP
Fraction Gag-Ub
1
0.5
0.25
0.05
0.01
0
24 hr VLPs
48hr VLPs
Lysates
Gag-Ub
Gag

Ubiquitin-directed PFV budding is Class E pathway-dependent

Ubiquitin possesses several residues required for its polymerization and recognition by cellular signaling factors. Lys48 and Lys63 are involved in the formation of polyubiquitin chains, which target proteins for proteasomal degradation [262,263,264] or other regulatory events [264,265,266,267,268], respectively. Gln62 and Glu64 have been implicated in binding Tsg101 and its yeast homolog Vps23 [269]. Phe4 is important for ubiquitin-mediated receptor protein internalization [270,271]. Leu8 and Ile44 are key residues in a hydrophobic patch [271,272,273] that forms an essential interaction surface for numerous cellular proteins, including some VPS proteins (summarized in Table 5). Notably, overexpression of ubiquitin with mutated Phe4 and Ile44 residues has been shown to inhibit HIV-1 VLP production [273]. Mutagenic disruption of the Leu8/Ile44 hydrophobic patch strongly inhibited ubiquitin-dependent rescue and enhancement of PFV Gag budding, whereas the other residues appeared to be dispensable (Fig. 22A). Thus, ubiquitin seems to mediate VLP production by recruiting cellular factors to sites of virion assembly, in effect serving as a late domain.

The function of all conventional retroviral late domains requires an intact Class E VPS pathway [103,108,122,123,124]. Thus, if budding of ubiquitin-fused Gag occurs by the same mechanism as that of PPxY-containing Gag, it should be inhibited by disruption of the Class E machinery. Indeed, budding of Lck-PFV-ΔPSAP-Ub and Lck-PFV-PSAP-Ub was suppressed in a dose-dependent manner by overexpression of dominant negative Vps4 (E228Q) (Fig. 22B), indicating that ubiquitin can promote particle release only in cells with an intact Class E pathway.

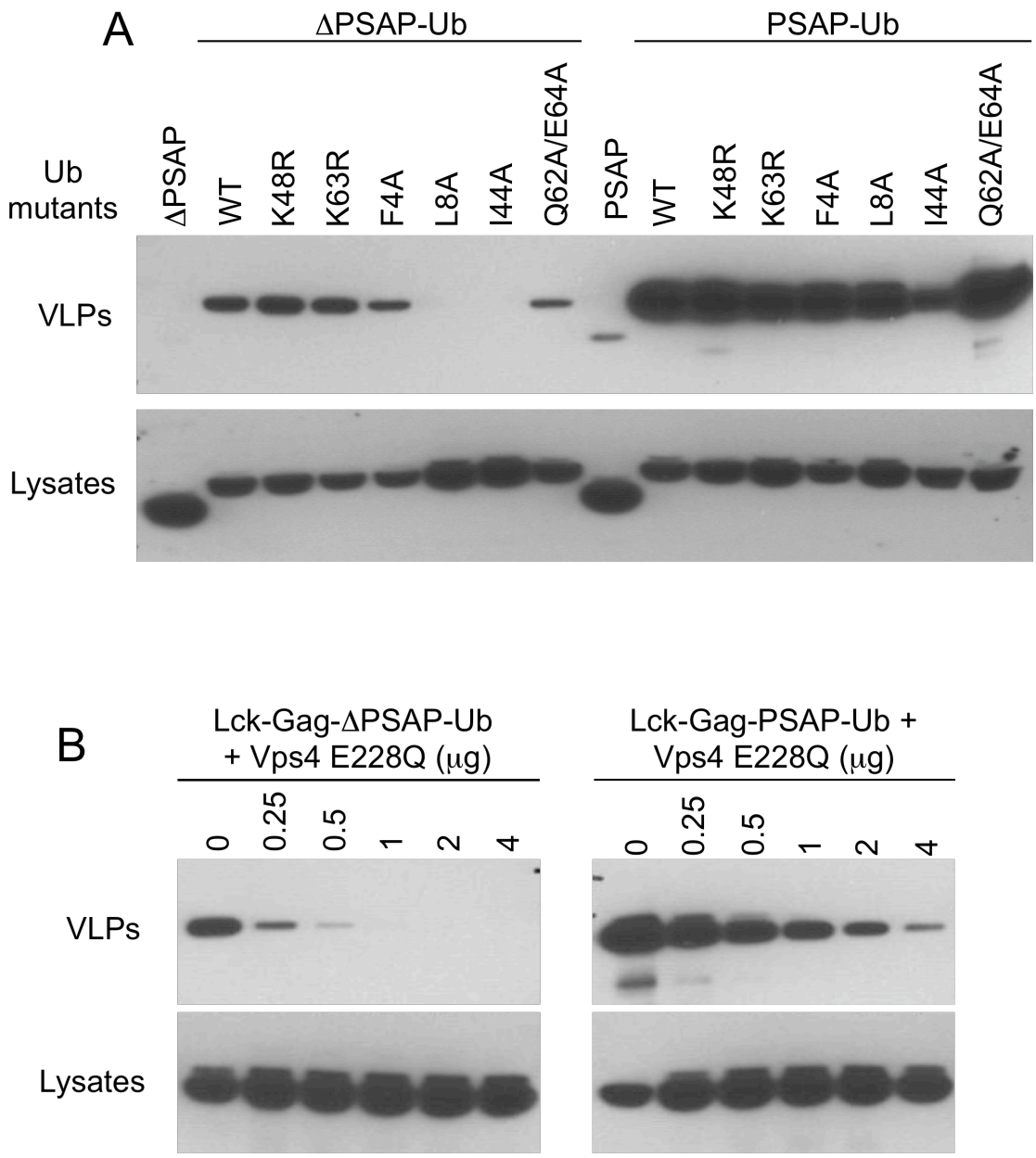


Figure 22. Ubiquitin-dependent VLP production requires Class E VPS machinery engagement. (A) VLP release from 293T cells expressing Lck-Gag-Ub containing either the wild-type or inactive late domain and the indicated mutations in ubiquitin. (B) VLP production from 293T cells expressing Lck-Gag-Ub containing either the wild-type or nonfunctional late domain and increasing amounts of GFP-Vps4 (E228Q).

Several Class E factors bind ubiquitin

Several Class E vacuolar protein sorting factors have been reported to encode ubiquitin interacting motifs (Table 5). Although the affinity of such domains for monoubiquitin is relatively weak (K_d >100μM), many Class E factors form multiprotein complexes that potentially contain several ubiquitin-binding surfaces, which could provide sufficient avidity for their interaction with ubiquitinated proteins. The efficiency with which ESCRT complexes are recruited to viral assembly sites would would likely be dependent on the concentration of ubiquitin in the vicinity of VLP assembly, which is consistent with our finding that a large fraction of Gag must carry ubiquitin to compensate for the absence of a late domain (Fig. 21D). We performed a yeast two-hybrid assay to survey the binding of various Class E factors to ubiquitin containing either an intact (WT) or disrupted hydrophobic patch (I44A). Several factors exhibited binding interactions above background levels (Fig. 23); however, only HBP/STAM, CIN85, ALIX, and UBPY binding appeared to require the Ile44 hydrophobic patch. We note that multiple subunits of the ESCRT-I complex have been reported to bind ubiquitin or have domains that are predicted to bind ubiquitin [103,147,148,257,274], and while we were not able to detect interactions with individual subunits in out yeast two hybrid assays, it is quite possible, and even likely, that the complete ESCRT-I complex binds ubiquitin.

Table 5. Mammalian Class E factors encoding ubiquitin binding domains

Class E factor	Domain type	Kd	Methods	References
Tsg101	UEV	~500μM	SPR, NMR, structure	[103,147,148,274]
Vps37A	putative UEV		sequence	[257]
Hrs	DUIM, VHS	~300μM, ~1.4mM	IP, SPR, structure	[161,274,275,276,277, 278,279]
STAM	UIM, VHS	~430μM, ~220μM	IP, NMR	[155,160,161,280]
Eap45	GLUE	~300μM	IP, SPR, structure	[166,281,282]
Eps15	UIM		IP, SPR	[277,279]
ALIX	Unknown		IP	[258]
CIN85	SH3		IP, NMR	[283]

SPR: surface plasmon resonance, NMR: nuclear magnetic resonance

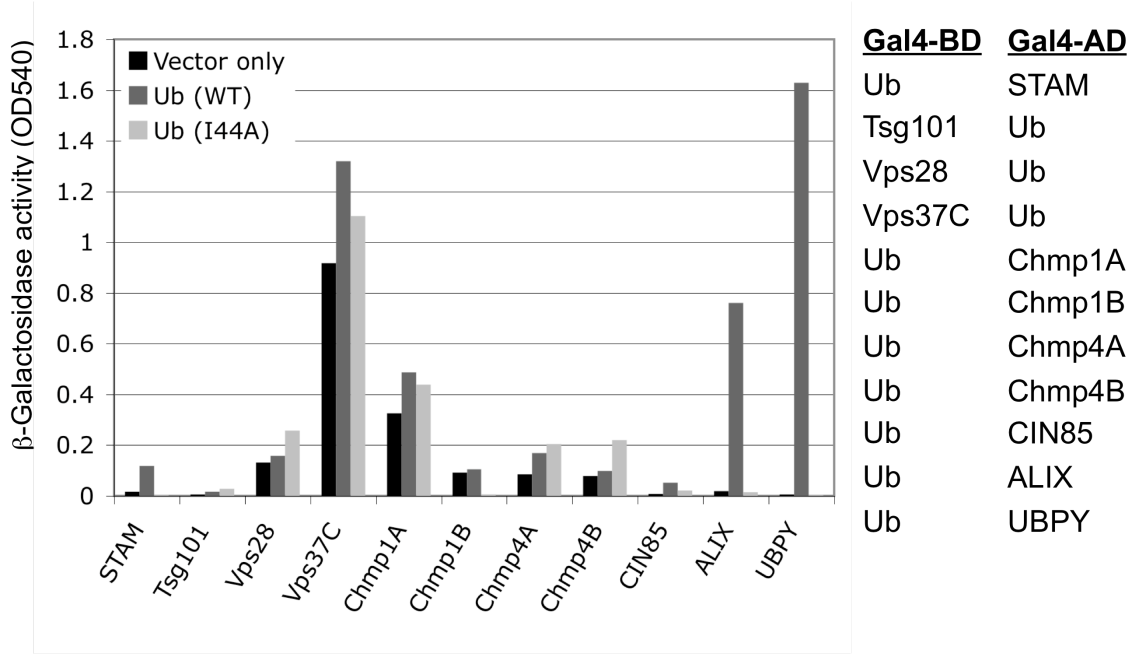


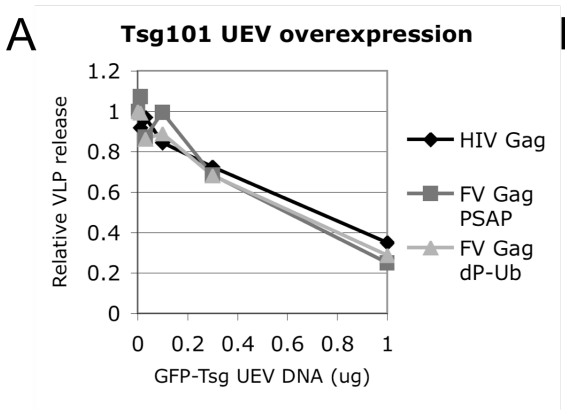
Figure 23. Several Class E vacuolar protein sorting factors interact with ubiquitin in a hydrophobic patch-dependent manner. Yeast two-hybrid analysis of the interaction between the indicated Class E factors and ubiquitin containing either an intact (Ub WT) or disrupted (Ub I44A) hydrophobic patch. β-galactosidase expression was measured as optical density at 540nm (OD540) in Y190 cells transformed with the indicated Gal4-DNA binding domain (Gal4-BD) or activation domain (Gal4-AD) fusion constructs.

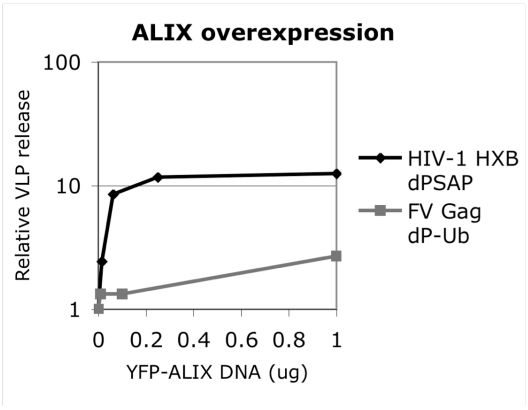
Perturbation of Class E factor function modestly affects ubiquitin-dependent budding

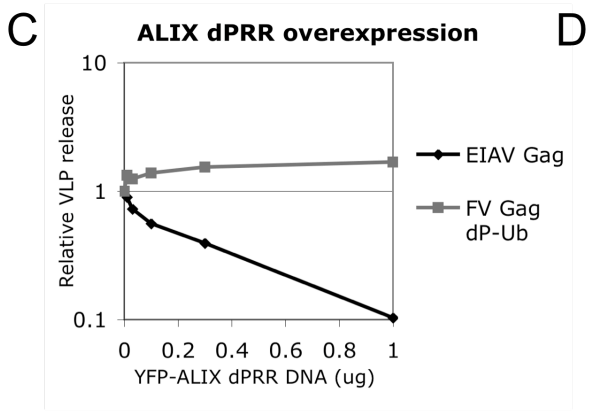
We sought to determine the sensitivity of ubiquitin-dependent rescue of PFV budding to perturbation of different branches of the Class E pathway. Therefore, we tested the efficiency of Lck-PFV Gag-ΔPSAP-Ub particle release in cells overexpressing either full length or putatively dominant negative fragments of several Class E factors with reported ubiquitin binding activity. Overexpression of the Tsg101 UEV domain, which binds ubiquitin but lacks the ability to recruit downstream components of the ESCRT pathway, inhibited ubiquitin-dependent budding of Lck-PFV Gag to a similar extent as P(T/S)AP-dependent budding of Lck-PFV Gag and HIV-1 Gag (about 3-fold, Fig. 24A). However, mutation of the ubiquitin-binding site of TsgUEV (N45A, [147,148]) had no effect on its ability to inhibit Lck-PFV budding (data not shown). Since the UEV domain interacts with PT/SAP motifs as well as ubiquitin, the N45A mutant UEV could, in theory, be recruited to Gag-ubiquitin assembly sites indirectly, by binding ESCRT-0 through the PSAP motif in Hrs.

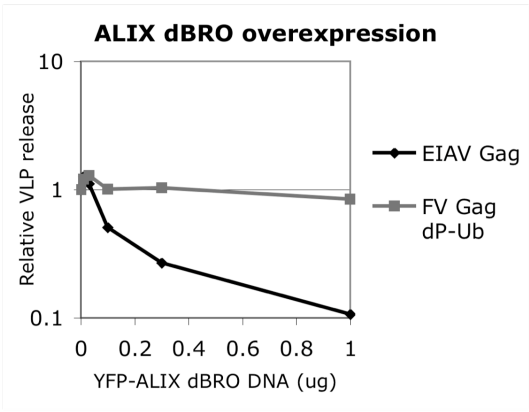
PSAP- and ubiquitin-dependent PFV Gag budding were both modestly inhibited by overexpression of Hrs, containing either a wild-type or mutated (LA265,266AL) ubiquitin binding pocket [159,277] (Fig. 24E and data not shown). Endogenous Hrs localizes to early endosomes, and its overexpression causes Tsg101 redistribution from late to early endosomes [158]. Moreover, overexpression of Hrs has been shown to inhibit HIV-1 budding by a mechanism that requires its interaction with Tsg101 [284]. Overexpressed Hrs may, therefore, inhibit ubiquitin-dependent budding, independently of

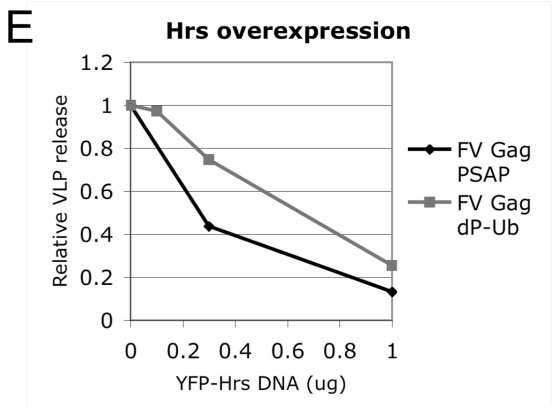
Figure 24. Effect of Class E factor overexpression on ubiquitin-dependent budding of late domain-deficient PFV Gag. Quantitative Western blot analysis of relative VLP production by 293T cells expressing Lck-PFV Gag-ΔPSAP-Ub and increasing quantities of GFP-Tsg101UEV (A), YFP-ALIX (B), YFP-ALIX-ΔPRR (C), YFP-ALIX-ΔBro (D), YFP-Hrs (E), YFP-UBPY (F), or YFP-Eap45 (G). Effect of factor overexpression on VLP release from 293T cells expressing Lck-PFV Gag-PSAP (A, E, and G), HIV-1 Gag (A), HIV-1 HXB-ΔPSAP proviral plasmid (B), and EIAV Gag (C and D) is shown for comparison.

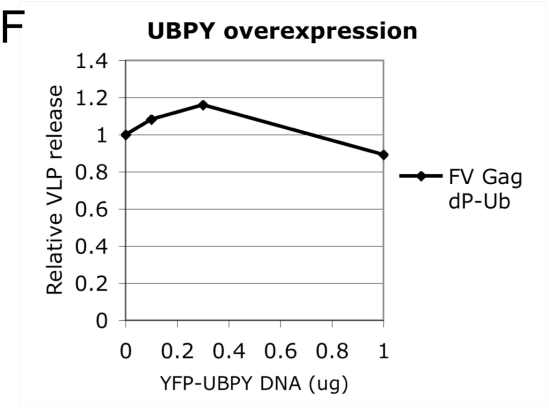

A
Tsg101 UEV overexpression
Relative VLP release
1.2
1
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1
GFP-Tsg UEV DNA (ug)
HIV Gag
FV Gag PSAP
FV Gag dP-Ub

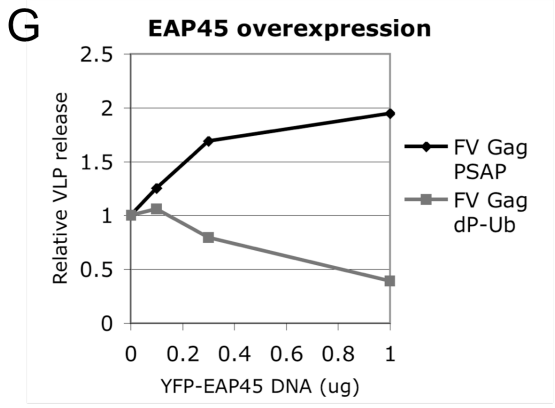

B
ALIX overexpression
Relative VLP release
100
10
1
0 0.2 0.4 0.6 0.8 1
YFP-ALIX DNA (ug)
HIV-1 HXB dPSAP
FV Gag dP-Ub


C
ALIX dPRR overexpression
Relative VLP release
10
1
0.1
0 0.2 0.4 0.6 0.8 1
YFP-ALIX dPRR DNA (ug)
EIAV Gag
FV Gag dP-Ub


D
ALIX dBRO overexpression
Relative VLP release
10
1
0.1
0 0.2 0.4 0.6 0.8 1
YFP-ALIX dBRO DNA (ug)
EIAV Gag
FV Gag dP-Ub


E
Hrs overexpression
Relative VLP release
1.2
1
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1
YFP-Hrs DNA (ug)
FV Gag PSAP
FV Gag dP-Ub


F
UBPY overexpression
Relative VLP release
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 0.2 0.4 0.6 0.8 1
YFP-UBPY DNA (ug)
FV Gag dP-Ub


G
EAP45 overexpression
Relative VLP release
2.5
2
1.5
1
0.5
0
0 0.2 0.4 0.6 0.8 1
YFP-EAP45 DNA (ug)
FV Gag PSAP
FV Gag dP-Ub

its own ability to interact with ubiquitin, by sequestering endogenous Tsg101 away from sites of virus particle assembly.

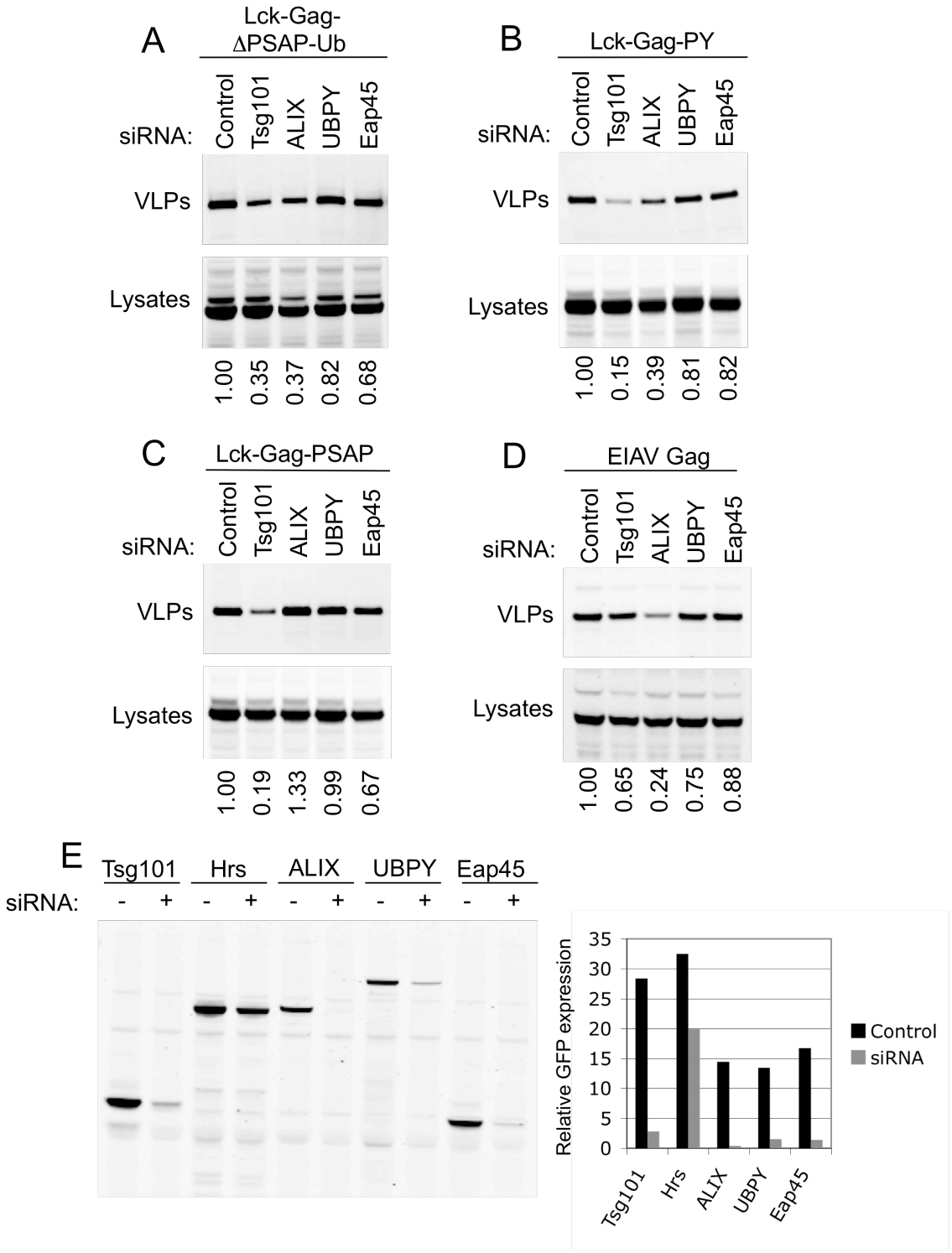
Expression of full length ALIX at concentrations that strongly stimulated budding of HIV-1 encoding a mutated PTAP motif [196] marginally enhanced ubiquitin-dependent Lck-PFV Gag budding (Fig. 24B). Converesely, overexpression of ALIX fragments lacking either the Bro1 domain or the proline-rich region, which are important for interactions with CHMP4 and Tsg101, respectively [108,122,123,197,198], exhibited a strong dominant negative effect on YPDL-dependent EIAV budding and had no effect on Lck-PFV Gag-ΔPSAP-Ub budding (Fig. 24C, D).

Overexpression of the wild-type and catalytically inactive deubiquitninating enzyme UBPY had no appreciable effect on ubiquitin-dependent VLP production (Fig. 24F and data not shown). Overexpression of the ESCRT-II protein EAP45 slightly inhibited ubiquitin-dependent Lck-PFV Gag budding, and the inhibition was unaffected by mutations in the ubiquitin binding site of EAP45 (VFE67,68,70AAA [281,282]) (Fig. 24G and data not shown).

Inhibition of ubiquitin-dependent budding by Class E factor depletion

Overall, the effects of ESCRT protein overexpression on ubiquitin dependent budding were rather modest and difficult to interpret. Therefore, We next determined the effect of siRNA depletion of ubiquitin-binding Class E factors on Lck-PFV Gag budding. Due to the inefficient knockdown of Hrs (Fig. 25E), its effect on budding could not be reliably assessed. We did not test the effect of STAM depletion, since it cannot associate with membranes or interact with the ESCRT pathway in the absence of Hrs [155,158],

Figure 25. Inhibition of Lck-PFV Gag budding by Class E factor depletion is late domain-specific. (A-D) Quantitative Western blot analysis of VLP production from 293T cells expressing Lck-PFV Gag-ΔPSAP-Ub (A), Lck-PFV Gag-PY (B), Lck-PFV Gag-PSAP (C), or EIAV Gag (D) and siRNAs directed against the indicated Class E factors. (E) Quantitative Western blot analysis of siRNA Class E factor depletion. Lysates of 293T cells transfected with GFP-Tsg101 or YFP-Hrs, -ALIX, -UBPY, or -Eap45 expression plasmids and siRNAs targeting either luciferase (Control) or the specified Class E factors were probed with αGFP monoclonal antibody.


A
Lck-Gag-ΔPSAP-Ub
siRNA:
Control
Tsg101
ALIX
UBPY
Eap45
VLPs
Lysates
1.00
0.35
0.37
0.82
0.68
B
Lck-Gag-PY
siRNA:
Control
Tsg101
ALIX
UBPY
Eap45
VLPs
Lysates
1.00
0.15
0.39
0.81
0.82
C
Lck-Gag-PSAP
siRNA:
Control
Tsg101
ALIX
UBPY
Eap45
VLPs
Lysates
1.00
0.19
1.33
0.99
0.67
D
EIAV Gag
siRNA:
Control
Tsg101
ALIX
UBPY
Eap45
VLPs
Lysates
1.00
0.65
0.24
0.75
0.88
E
Tsg101
Hrs
ALIX
UBPY
Eap45
siRNA:
- + - + - + - + - +
Relative GFP expression
35
30
25
20
15
10
5
0
Tsg101
Hrs
ALIX
UBPY
Eap45
Control
siRNA

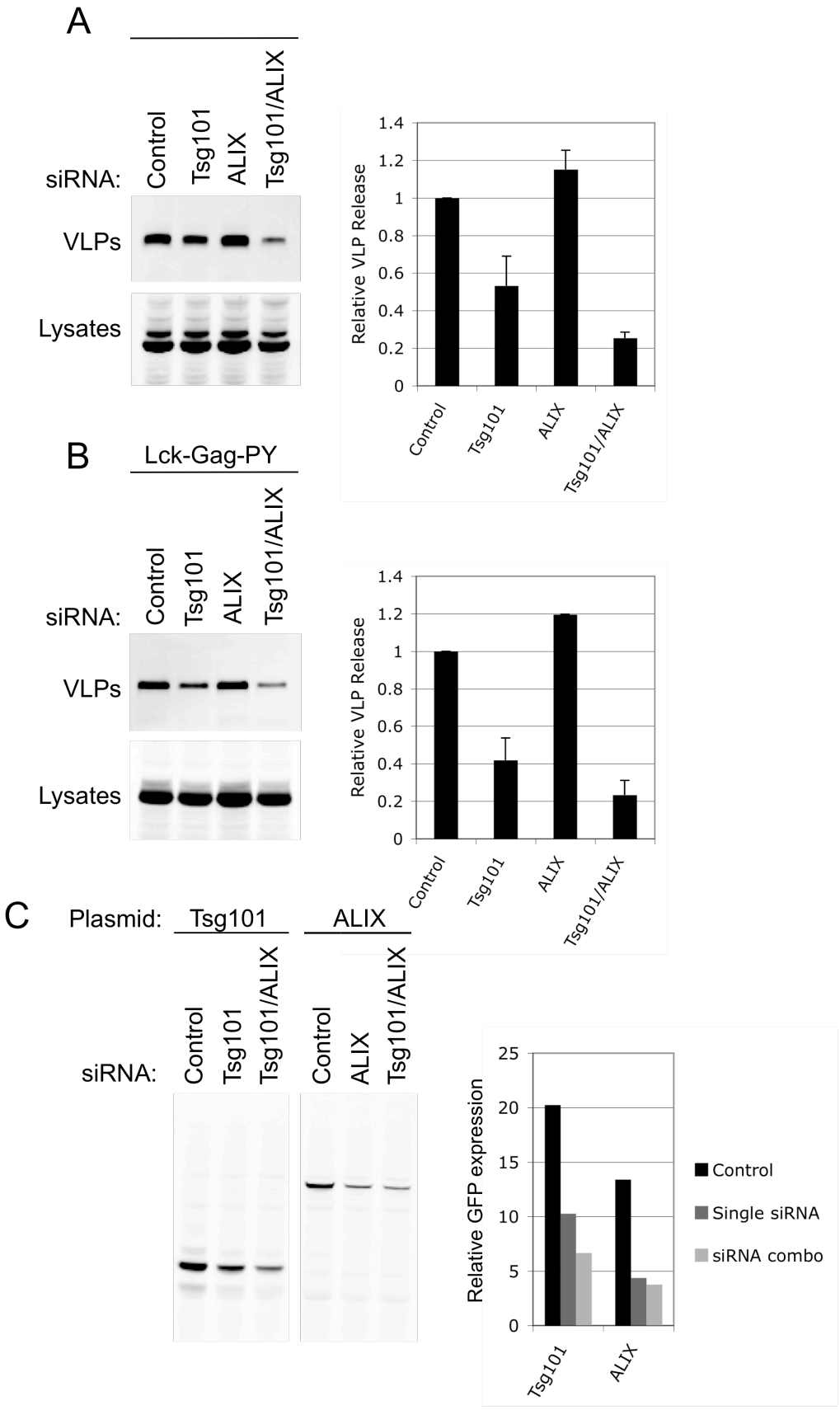
and thus appears to play an auxiliary role in ubiquitinated cargo recognition. Nevertheless, ubiquitin-dependent budding was modestly inhibited by depletion of Tsg101 or ALIX (2-3-fold) but not by depletion of UBPY or EAP45 (Fig. 25A). PPxY-dependent budding of PFV Gag exhibited a similar pattern of inhibition by Class E factor knockdown but appeared to be more sensitive to Tsg101 depletion (Fig. 25B). In contrast, PSAP-dependent Lck-PFV Gag budding was specifically inhibited by depletion of Tsg101 (over 5-fold inhibition, Fig. 25C) and EIAV Gag budding was specifically inhibited by depletion of ALIX (about 4-fold inhibition, Fig. 25D).

Several Class E factors may serve redundant roles in recognizing ubiquitinated species and mediating ubiquitin-dependent virus particle release. Thus, we sought to determine whether simultaneous depletion of multiple factors, specifically Tsg101 and ALIX, would exhibit a stronger inhibitory effect on budding than depletion of individual factors. Class E factor knockdown was significantly less efficient under the conditions used for this assay (Fig. 26C), which may account for the lack of inhibition of VLP release by expression of siRNAs targeting ALIX. Nevertheless, cotransfection of Tsg101 and ALIX siRNAs together had a significantly stronger inhibitory effect on both ubiquitin- and PPxY-dependent Lck-PFV Gag budding than expression of either siRNA alone (Fig. 26A, B).

Summary

We generated ubiquitin-fused PFV Gag proteins to mimic ubiquitin deposition at viral assembly sites in the absence of HECT domain recruitment. Remarkably, direct fusion of ubiquitin to the carboxy terminus of PFV Gag enabled budding and in the

Figure 26. Depletion of multiple Class E factors enhances VLP release inhibition. (A and B) Quantitative Western blot analysis of VLP production from 293T cells expressing Lck-PFV Gag-ΔPSAP-Ub (A) or Lck-PFV Gag-PY (B) and siRNAs targeting the indicated Class E factors. Cells transfected with a single Class E factor siRNA were co-transfected with an equal amount of control siRNA to keep total siRNA levels constant. (C) Quantitative Western blot analysis of ectopically expressed fluorescent protein-fused Class E factor depletion in 293T cells transfected with multiple siRNAs. Cell lysates were probed with αGFP monoclonal antibody.


A
siRNA:
Control
Tsg101
ALIX
Tsg101/ALIX
VLPs
Lysates
Relative VLP Release
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Tsg101
ALIX
Tsg101/ALIX
B
Lck-Gag-PY
siRNA:
Control
Tsg101
ALIX
Tsg101/ALIX
VLPs
Lysates
Relative VLP Release
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
Tsg101
ALIX
Tsg101/ALIX
C
Plasmid:
Tsg101
ALIX
siRNA:
Control
Tsg101
Tsg101/ALIX
Control
ALIX
Tsg101/ALIX
Relative GFP expression
25
20
15
10
5
0
Tsg101
ALIX
Control
Single siRNA
siRNA combo

absence of a conventioan L-domain, and potently stimulated PSAP-dependent budding. Ubiquitin-dependent particle production was abolished by overexpression of dominant negative Vps4 and diminished by mutations within the ubiquitin hydrophobic patch. Depletion of individual class E factors partially suppressed ubiquitin-dependent budding, and the effect was enhanced by simultaneous depletion of Tsg101 and ALIX, suggesting that their interactions with ubiquitin provide redundant mechanisms for VPS pathway engagement.

Chapter VI. Discussion

Efficient separation of nascent virions from the plasma membrane requires engagement of the cellular Class E VPS pathway by late domains, short peptide motifs encoded within viral structural proteins. PT/SAP and YPxL/LxLF-type late domains recruit VPS factors with well-documented physical links to the ESCRT-III complex of the pathway, which is thought execute the membrane scission event critical for release of virons. On the other hand, PPxY late domains recruit Nedd4-like HECT ubiquitin ligases, which require their catalytic domains and an intact Class E pathway for their function in virus release. However, the mechanism of Class E machinery engagement by HECT ligases is poorly understood. Moreover, ubiquitin plays a general role in late domain-dependent budding, as its depletion arrests the release of viruses that utilize different late domains at a late stage of budding, but its precise function is unclear.

The major structural proteins of several retroviruses become ubiquitinated as a consequence of late domain activity; however, it is unclear whether these modifications play a functional role in promoting virion release or if they are merely bystander reactions of promiscuous ubiquitin ligases. Two studies have shown that mutation of multiple lysine residues in HIV-1 and RSV Gag significantly inhibits particle production [237,238]. However, the budding defects observed in these studies may have been due to structural disruptions in Gag rather than the loss of ubiquitin acceptor sites. We took advantage of a naturally occurring lysine-poor Gag protein (PFV Gag) to generate a lysine free Gag that could direct the assembly of virus-like particles at the plasma membrane in the absence of other viral protein expression. Lysine free PFV Gag

supported efficient PSAP late domain-dependent particle release. Remarkably, direct ubiquitination of PFV Gag was dispensable for the function of the PPxY late domain, which nevertheless recruited HECT ligases and required their catalytic activity to promote budding.

Similar observations have been made with regard to vacuolar sorting of certain proteins in yeast. For example, recruitment of the yeast HECT ligase Rsp5 by Sna3, through a PPxY motif in its cytoplasmic tail, promotes Sna3 polyubiquitination and sorting into the MVB pathway. However, sorting of lysine free Sna3, which does not get ubiquitinated, still requires the catalytic activity of Rsp5 [225,226,227]. Thus, cargo protein ubiquitination is not universally required for ubiquitin ligase-dependent endosomal sorting using the VPS pathway.

In theory, HECT ligases could stimulate viral particle release via the Class E pathway by numerous mechanisms other than direct viral protein ubiquitination. The ligases themselves could function as scaffolds for VPS protein recruitment, either directly or through unidentified bridging factors. Our lab has previously shown that the isolated HECT domain of WWP1 can behave in soluble Class E factor-like manner, in that it is retained on aberrant endosomal compartments induced by overexpression of catalytically inactive Vps4 (E228Q) [115]. Moreover, physical interactions between Class E factors and HECT ligases have been reported, albeit not necessarily via the HECT domain. Specifically, Tsg101 can associate with a fragment of Nedd4 containing the C2 and WW domains [285], and Hrs can associate with and become ubiquitinated by Itch [286]. Alternatively, HECT ligase-mediated ubiquitination could modulate the stability, activity, or interactions of specific Class E factors or associated proteins. Indeed, several factors

involved in yeast and human endosomal trafficking can be ubiquitinated by HECT ligases [286,287]. Finally, since several Class E factors that function early in the pathway contain ubiquitin-binding domains, HECT ligase-mediated autoubiquitination, or ubiquitination of trans-acting factors, may serve to concentrate ubiquitinated species at virion production sites, thereby nucleating the assembly of the Class E machinery.

Since the catalytic domains of HECT ligases are critical for their late domain-stimulating ability, we compared the activities of HECT domains from different members of the human Nedd4-like family of HECT ubiquitin ligases. We found the catalytic cysteine residue to be strictly required for stimulation of PPxY-dependent budding of lysine-free PFV Gag. Meanwhile, HECT ligase-mediated stimulation of VLP release did not correlate with HECT domain recruitment to mutant Vps4-induced Class E compartments. Thus, the ubiquitin conjugating activity of HECT domains appears to be critical for their role in particle release, even in the absence of direct Gag ubiquitination. This is consistent with recent findings that ISG15, a ubiquitin-like molecule, can inhibit PPxY-dependent release of vesicular stomatitis virus (VSV) and Ebola virus VP40 VLPs by suppressing the catalytic activity of Nedd4 and several related ubiquitin E3 ligases [228,229]. It is important to note that ISG15 inhibits Nedd4 autoubiquitination, and not merely viral protein ubiquitination, by blocking the association of Nedd4 with E2 enzymes and impairing the formation of thioester-bound HECT-Ub intermediates [228].

Catalytically active HECT domains varied in their ability to stimulate PPxY-dependent particle release. Notably, chimeric HECT ligases exhibited similar patterns of stimulation of PPxY-dependent VLP production by lysine-free and lysine-containing PFV Gag (data not shown). We found this variability to correlate directly with the levels

of HECT ligase autoubiquitination, but not with their ability to ubiquitinate a Gag substrate that contains several lysines in close proximity to a PPxY motif. This is in agreement with our finding that direct Gag ubiquitination is dispensable for PPxY-dependent budding and consistent with a model in which HECT ligases promote VLP release by ubiquitinating trans-acting factors. However, it is unclear whether ubiquitination of HECT ligases, specifically, is critical for Class E pathway engagement, or if autoubiquitinated ligases simply represent the one of the ubiquitinated protein species at virion assembly sites.

The C2 and WW domains appear to be dispensable for HECT ligase-mediated VLP release. Catalytically active HECT domains fused to the Tsg101 UEV domain stimulated PSAP-dependent budding, while the corresponding catalytically inactive mutants suppressed particle production. Thus, as long as they are enzymatically intact and can be recruited to sites of virion assembly, HECT domains encode all of the activities necessary for the promotion of VLP release. It seems unlikely, therefore, that HECT ligases engage the Class E pathway by recruiting substrates via their WW or C2 domains.

The autoubiquitination activity of HECT E3 ligases has been linked to their ability to monoubiquitinate substrates by a mechanism called coupled ubiquitination [288]. Conventional HECT ligase substrate recognition occurs via relatively stable interactions between the WW domains of the ligase and the PPxY motifs in the substrate. In contrast, proteins that contain ubiquitin interacting motifs (UIMs), sometimes referred to as “ubiquitin receptors”, can transiently associate with autoubiquitinated HECT ligases and become monoubiquitinated in the absence of WW-PPxY interactions. Thus, it is

conceivable for autoubiquitinated HECT ligases to promote VLP release by recruiting UIM-containing VPS proteins to sites of virion assembly. Interestingly, the catalytically active WWP2 HECT ligase, which does not exhibit a significant amount of autoubiquitination, cannot ubiquitinate UIM-containing substrates, presumably because it cannot efficiently recruit them [288]. Similarly, the weak autoubiquitination of our chimeric WWP1/WWP2 HECT ligase could account for its inability to engage the Class E machinery and thereby stimulate PPxY dependent PFV Gag budding in our experimental system.

Remarkably, direct fusion of a single ubiquitin moiety to the C-terminus of PFV Gag potently stimulated late domain-dependent and -independent budding. Direct ubiquitin fusion to RSV Gag has previously been shown to rescue the late budding defect in cells treated with proteasome inhibitors [104]. However, ubiquitin fusion cannot compensate for the deletion of the RSV late domain or further stimulate wild-type RSV Gag budding. Additionally, ubiquitin fusion rescues budding of EIAV Gag with an inactive late domain but has no effect on late domain-dependent EIAV Gag budding [258]. To our knowledge, our study is the first to demonstrate strong enhancement of late domain-dependent VLP production by direct fusion of ubiquitin to Gag. This could be attributed to inherently inefficient cofactor recruitment by the natural PFV late domain. Ubiquitin may serve as an additional docking site for VPS factors, thereby synergizing with the late domain to optimally utilize the available cellular Class E machinery.

Although we have not visually ascertained that the PFV Gag-ubiquitin fusion protein can assemble into bona fide VLPs, ubiquitin-dependent particle release occurred by a regulated mechanism. The Ile44 hydrophobic patch of ubiquitin, which is an

important interaction surface for various ubiquitin-binding domains (e.g. UIM, UBD, GLUE, etc.), was essential for the rescue of late domain-deficient Gag budding. Additionally, ablation of Class E machinery function by overexpression of catalytically inactive Vps4 suppressed ubiquitin-dependent particle production. Moreover, particles formed by ubiquitin-fused EIAV Gag are morphologically similar to those assembled by wild-type EIAV Gag [258].

Since ubiquitin-binding domains have a very low affinity for individual ubiquitin molecules and thus primarily mediate transient interactions, efficient retention of UIM-encoding Class E factors in the vicinity of Gag assembly sites would require an abundance of UIM docking sites. Indeed, the five ubiquitin binding domains (two VHSs and three UIMs) of ESCRT-0 have been shown to cooperatively bind polyubiquitin, but not mono- or diubiquitin, with high avidity, requiring at least three functional ubiquitin binding sites to support detectable levels of cargo sorting [161]. In agreement with this model, direct ubiquitin fusion to PFV Gag could effectively bypass the need for late domain-dependent recruitment of Class E factors only when a large fraction (~50%) of Gag molecules carried ubiquitin. This equates to about 1000 or more ubiquitin monomers per virion, assuming that ubiquitin-fused and unfused Gag molecules are incorporated into assembling particles with equivalent efficiency.

As with conventional late domain-dependent particle production, the Class E VPS pathway must be intact to support ubiquitin-dependent budding. Ablation of individual segments of the pathway resulted in partial inhibition of ubiquitin- and PPxY-dependent particle release. However, simultaneous depletion of Tsg101 and ALIX caused a stronger suppression of particle release than depletion of either factor alone, suggesting that they

may serve redundant roles in ubiquitin-dependent budding. Multiple ubiquitin-interacting domains within ESCRT-0, ESCRT-I, ESCRT-II and associated factors could, in principle, provide parallel mechanisms for ubiquitin-dependent engagement of the VPS machinery. Indeed, this was recently demonstrated in yeast, where simultaneous disruption of ubiquitin binding by ESCRT-I, ESCRT-II and Bro1 (ALIX homolog) and the association between ESCRT-I and ESCRT-0 was necessary to completely block the sorting of ubiquitinated cargo to the lysosome [154]. This suggests that all entry points into the Class E pathway must be simultaneously ablated to completely inhibit ubiquitin- or HECT ligase-dependent budding.

Future directions

Although we have not performed a detailed analysis of the specific types of ubiquitin modifications generated by our chimeric HECT ligases, we noticed variability in the distribution of synthesized chain lengths. For instance, WWP1/Itch preferentially formed longer polyubiquitin chains, whereas WWP1/Bul2 and WWP1/Rsp5 formed relatively high proportions of mono- or diubiquitinated products. These differences could contribute to the relative efficiency with which HECT ligases stimulate virus particle production. Moreover, the ubiquitin chains synthesized by various ligases may differ in the types of lysine linkages they contain. Full-length Rsp5, Nedd4, and Itch ligases have been shown to form Lys63-linked chains more efficiently than other types of polyubiquitin chains [289], but the linkage specificity of other Nedd4 family members has not been reported. The multivalent ESCRT-0 complex binds Lys63-linked ubiquitin chains with greater affinity than Lys48-linked or linear head-to-tail ubiquitin chains

[161]. Thus, chain length and linkage specificity could influence the ability of ubiquitin ligases to engage the Class E machinery.

Several aspects of HECT domain biochemistry are amenable to experimental manipulation. We can force certain types of ubiquitin chain linkages to be overrepresented or excluded in ubiquitin transfer or VLP production assays by overexpression of ubiquitin mutants that either contain or lack specific lysine residues. Thus, we could assess the chain type specificity of our chimeric HECT ligases and determine whether certain ubiquitin linkage types are preferentially recognized by the VPS machinery to promote virus particle release. The specificity for Lys48 or Lys63-type linkages is thought to be determined by a 60-residue segment at the HECT domain C-terminus [289]. We could thus, in principle, assess the relative importance of ubiquitin linkages in viral budding by swapping this domain between HECT ligases with different linkage specificities. Since all of the Nedd4-like HECT domains tested thus far preferentially generate Lys63-linked ubiquitin chains, it would be interesting to test the ability of the E6AP HECT domain, which preferentially forms Lys48-linked chains [289], to stimulate PPxY-dependent VLP release.

HECT domains possess additional mechanisms that regulate their enzymatic activity. For example, the highly conserved α1 helix suppresses the overall catalytic activity of HECT domains and preferentially inhibits their autoubiquitination [290]. Disruption of interactions between the α1 helix and the HECT domain N-lobe may increase HECT ligase promiscuity and have interesting implications for their ability to promote VLP release. Additionally, the N-lobe of HECT domains contains a noncovalent ubiquitin binding site, which enables polyubiquitination by some HECT ligases and

limits the length of ubiquitin chains produced by others [213,214]. Thus, it would be interesting to test the effect of ubiquitin binding site disruption on the ubiquitination and particle release stimulation patterns of our chimeric HECT ligases.

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