Endosomal Sorting Complex Required for Transport (ESCRT) Complexes Induce Phase-separated Microdomains in Supported Lipid Bilayers*

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The endosomal sorting complex required for transport (ESCRT) system traffics ubiquitinated cargo to lysosomes via an unusual membrane budding reaction that is directed away from the cytosol. Here, we show that human ESCRT-II self-assembles into clusters of 10–100 molecules on supported lipid bilayers. The ESCRT-II clusters are functional in that they bind to ubiquitin and the ESCRT-III subunit VPS20 and exclude an Lα-phase-specific dye. These results show that ESCRT complexes are capable of inducing lateral lipid phase separation under conditions where the lipids themselves do not spontaneously phase-separate. This property could facilitate ESCRT-mediated membrane budding.

The ESCRTs are complexes of sorted from yeast to humans and play key roles in receptor down-regulation and lysosome biogenesis (1–3), the budding of HIV-1 and other membrane-enveloped viruses, and cytokinesis (4), among other cellular pathways. ESCRTs function in receptor down-regulation and endosome biogenesis by sorting ubiquitinated membrane proteins into nascent membrane buds that evade away from the cytosol, and a parallel pathway is directly involved in budding from the plasma membrane (5). The topology of the ESCRT membrane budding reaction is opposite to that of the well known coated vesicle budding pathways (6, 7). The ESCRT budding topology is, however, the same as that of several lipid-driven pathways (8). These include virus- and toxin-driven clustering of the lipids GM1 and Gb3 (9), ceramide-dependent exosome budding (10), and lysobisphosphatidic acid-dependent budding into late endosomes (11). The key lipid requirement in the ESCRT-driven MVB biogenesis pathway is for phosphatidylinositol 3-phosphate (PI(3)P). PI(3)P is essential for the progression of MVB biogenesis because of its key role in the recruitment of ESCRT-0 and ESCRT-II to endosome membranes.

In vitro reconstitution of multivesicular body (MVB) biogenesis by the ESCRTs in a PI(3)P-containing giant unilamellar vesicle (GUV) model system suggested the following division of labor. ESCRT-I and -II form inward buds (12) by self-assembling together at the neck of the bud. Subsequently, ESCRT-III polymerizes on the membrane (13, 14) into structures that drive the final scission of the membrane bud (15, 16). A structural model for an ESCRT-I–II pore assembly at the bud neck was proposed on the basis of a low resolution structure of the supercomplex (17, 18), but the pathway for nucleation of the putative pore assembly is not clear. One prediction of the pore model is that ~6–10 ESCRT-II complexes should line the neck of the pore of the 25 nm buds in yeast MVBs, and therefore, twice that number should assemble when 50-nm buds are formed in human cells. However, the size of ESCRT clusters on membranes has not been measured directly.

The energetic driving force for bud formation and the factors controlling the size of the membrane bud are important questions in the ESCRT field. A computational analysis of this pathway suggested that the neck-based budding mechanism would...
be strongly favored if it were coupled to the existence of line tension. The magnitude of the line tension is a key element in determining the size of the bud in this model. Line tension would occur only if a distinct lipid domain were present in the bud. A key prediction of this model, which we sought to test, was that at least some of the ESCRT complexes should be capable of inducing lipid phase separation in membranes of the same compositions that give rise to membrane buds in GUVs.

As a model system, we chose human ESCRT-II, whose interactions with ESCRT-III, ubiquitin, and membranes are particularly well characterized. Here, we show that ESCRT-II self-assembles on supported lipid bilayers (SLBs). The SLB format was selected for this study because of its advantages as a substrate for quantitative analysis by total internal reflection fluorescence microscopy (TIRFM). These experiments allow the size of ESCRT clusters and the stoichiometry of their interactions to be directly measured, which has not been possible in other modalities used in this field. The ESCRT-II assemblies have the expected ability to bind membrane-tethered ubiquitin and the most upstream of the ESCRT-III subunits, VPS20. These binding events occur via the known structural interfaces, demonstrating that these assemblies recapitulate functional interactions. TIRFM analysis in the SLB format allows us to analyze the stoichiometry of these interactions on membranes with excellent precision. The self-assembly of ESCRT-II on SLBs requires the presence of cholesterol and leads to the induction of a liquid ordered (Lo)-like domain. This provides the first direct evidence that ESCRT complexes are capable of inducing lipid phase separation, which in turn could give rise to line tension and so enhance budding and scission.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Human ESCRT-III subunit VPS20 and its mutants were expressed and purified as described previously (19). Briefly, N-terminal His₆-maltose-binding protein-TEV cleavage site fusion proteins were expressed in E.coli BL21 Star at 30 °C overnight after induction with 0.5 mM isopropyl thiogalactoside at an absorbance (A₆₀₀ nm) of 0.8. The proteins were affinity-purified using Ni-NTA resin (Qiagen). VPS20 was cleaved using TEV protease and further purified by size exclusion chromatography on a Superdex 75 column (GE Healthcare). Full-length human ESCRT-II was purified from E.coli BL21 Star as described previously (20). Briefly, all three subunits were co-expressed from pST39 vector with a His₆ tag and TEV cleavage site fused to the C-terminus of Cys-Ala-His6, ubiquitin-Cys-Ala-His6 or ubiquitin-144D-Cys-Ala-His6) were incubated overnight at 4 °C with a 20-fold molar excess of Alexa488, Atto488, Atto647, or rhodamine maleimide derivatives. Labeled maltose-binding protein VPS20 was diluted to 1 μM and cleaved by TEV protease. After cleavage, the protease was removed using Ni-NTA resin (Qiagen). The labeling efficiency estimated spectroscopically was ~100%. Wild-type human and yeast ESCRT-II, which contain multiple native Cys, were labeled by the same procedure, except with a 2.5-fold molar excess of dye. For all samples, unreacted dye was removed by gel filtration chromatography on Superdex 75 or 200. The labeling efficiency estimated spectroscopically was 200% (Alexa488) and 180% (Atto647), respectively, and more than 90% of fluorescence signal was found to be in the VPS25 subunit as judged by SDS-polyacrylamide gel followed by analysis using Typhoon laser gel scanner (GE Healthcare). Labeled proteins were flash-frozen in liquid N₂ and stored at −80 °C until use.

**Preparation of Liposomes**—The following lipids were purchased from Avanti Polar Lipids: 1-palmitoyl-2-oleoyl-sn-glycerol-3-phospho-L-serine (POPS), 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (POPC), cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-lysine rhodamine B sulfonyl. Dipalmitoyl phosphatidylinositol 3-phosphate (DPP3(3)P) was purchased from Echelon, and dioleoyl phosphatidylinositol 3-phosphate was purchased from Avanti Polar Lipids. One mg of lipid mixture containing POPS (62 mol %), POPC (150 mol %), cholesterol (25 mol %), and P(3)P (3 mol %) was dried onto the borosilicate glass tube wall with a nitrogen stream. DPPI(3)P was used in all studies except where noted otherwise. For mixtures at a lower mole fraction of cholesterol, the cholesterol was replaced with POPC on an equimolar basis. The tube was dried for 3 h in a vacuum oven without heating and hydrated with buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 3 mM β-mercaptoethanol, 0.16% sodium ascorbate) overnight at 4 °C. The tube was vortexed and sonicated in a cup horn sonicator (Misonix) with room temperature water circulation until the small unilamellar vesicle (SUV) solution became clear (~50 watts for 10 min). The SUV suspension was freshly prepared on the day of the experiment. MgCl₂ was added to the SUV suspension to a final concentration of 5 mM just before loading into the flow cell. The flow cell was slowly loaded with the SUV suspension, incubated at room temperature for approximately 1 h, and washed with buffer.

**Flow Cell, Microscope, Camera, Illumination, and Data Processing**—Flow cell preparation, microscopy setup, and protein surface concentration measurements were as described previously (21). The 25-µm deep flow cells with inlet and outlet were assembled on fused silica slides. The slides were washed with Hellmanex II soap, rinsed extensively with deionized water, and air-dried for at least 2 h. A total internal reflection fluorescence inverted microscope with Nikon Plan Apo VC ×100 NA = 1.40 oil immersion objective, DualView (Photometrics) adapter with dcrxs630 dichroic, and an Andor iXon DU-897E camera were used for imaging. The spatial resolution...
of the camera was 6 pixels/μm. The presented images are 200 × 200 pixels or 33 × 33 μm. The s-polarized evanescent wave was created near the surface of flow cells by 488 and 633 nm laser beams normally incident to 70° fused silica dove prism. The illumination laser lines were blocked between the objective and camera with Semrock LineStop filters (A > 6). The laser power was controlled with calibrated attenuators. The camera signal was normalized according to the power and camera settings (reproducibility of the data were verified for different camera and power settings). Measured fluorescence intensity was proportional to the volume concentration of fluorescent molecules multiplied by the penetration depth of the evanescent wave (128 nm for 488-nm beam) plus the surface concentration of fluorescent molecules. The volume contribution to the fluorescence intensity signal was much smaller compared with the contribution of the surface for all ESCRT proteins at the concentration range used. The surface density of fluorescently labeled proteins was estimated by comparison between the fluorescent signals from the protein and a calibration measurement with fluorophore standard in the solution. The fluorophore standard had no detectable affinity to the lipid bilayer. The upper estimate of error for concentration measurements is 5 and 3% for fluorescence intensity measurements, and 2% is the day to day measured variance in laser excitation power. Taken together, we estimate that the number of bound ESCRT molecules has a combined error of ~14%. Metamorph 7 software was used for camera control and image acquisition. ImageJ was used for image analysis.

**RESULTS**

**ESCRT-II Forms Clusters on the Supported Lipid Bilayer**—We visualized the assembly of fluorescently labeled full-length recombinant human ESCRT-II complex on SLBs by TIRFM. ESCRT-II formed relatively small clusters on the SLB using a simple model membrane composition capable of supporting budding in GUV experiments. Conditions were kept near physiological with pH 7.4, and an ESCRT-II concentration at the low nanomolar level (Fig. 1A). The number of molecules was estimated from the intensity of the fluorescence. We standardized the amount of fluorescent signal per dye using free dye in solution. The number of dye molecules per cluster was computed, and from this the number of ESCRT-II complexes was obtained. The modal ESCRT-II cluster contains ~50 copies of the complex (Fig. 1B). We cannot rule out that this number may
be underestimated due to self-quenching effects. A range of sizes from ~10 to >100 is observed.

To probe whether there were pre-existing PI(3)P clusters or if generic PI(3)P binding proteins could induce clustering, the FYVE domain of EEA1 was flowed over the SLB. EEA1-FYVE bound but did not form clusters (Fig. 1B). Therefore, we ruled out the presence of pre-existing and nonspecific PI(3)P clustering, at least on the size scale observable by optical microscopy. Furthermore, the ESCRT-II sample in solution consists of non-aggregated singlet complexes as determined by size exclusion chromatography (data not shown). Because ESCRT-II behaves differently in this system from the generic PI(3)P-binding control, the cluster formation is best explained by the formation of specific lateral homotypic interactions on SLBs.

To compare these results to previous in vitro analysis of yeast ESCRTs on GUVs, the experiment was repeated with fluorescently labeled yeast ESCRT-II. Yeast ESCRT-II clusters do seem to be smaller and more numerous than human ESCRT-II. This probably explains why they appeared in the previous confocal study by Wollert and Hurley (12) as a continuum. We further examined whether human ESCRT-II formed clusters on GUVs, and we found that it does (Fig. 1E). The observation that human ESCRT-II clusters are clearly visible on confocal imaging of GUVs but the yeast clusters are not is explained by the finding that the human clusters are larger and but less numerous and hence better resolved from one another.

**ESCRT-II Clusters Bind Cargo**—To determine whether the ESCRT-II clusters corresponded to physiologically functional entities, we tested whether the clusters were capable of binding model cargo. To create model cargo tethered to the lipid bilayer, ubiquitin was fused to a C-terminal Cys and a His tag (ubiquitin-Cys-Ala-His subgroup). The single Cys was used to covalently attach the fluorescent dye Alexa488, and the labeled ubiquitin was tethered to the lipid bilayer via its His tag and the Ni²⁺ binding lipid DOGS-NTA. When tagged ubiquitin and ESCRT-II were passed together through the flow cell, ESCRT-II-ubiquitin co-clusters formed (Fig. 2, A–C). The ratio and absolute number of molecules were estimated from the fluorescence intensities, and the ratio was determined to be 1.09 ± 0.2 ESCRT-II/ubiquitin. The ubiquitin mutant I44D that does not bind ESCRT’s was used as a control for specificity (Fig. 2, D–F), demonstrating that the ESCRT-II clusters bind model cargo in a structurally specific manner.

**ESCRT-II Clusters Recruit the ESCRT-III Subunit VPS20**—To further test the physiological relevance of the observed ESCRT-II clusters, we probed whether they bound the immediate downstream ESCRT-III protein VPS20. We used two-color TIRFM to monitor the formation of clusters of fluorescently labeled ESCRT-II and VPS20 that were added to the flow cell together at 5 nM concentration (Fig. 3, A–C). VPS20 bound preferentially to the SLBs at the positions of the ESCRT-II clusters, without observably perturbing them. The VPS20¡ESCRT-II mutant lacking the ability to bind ESCRT-II was added to the flow cell in a separate experiment. VPS20¡ESCRT-II was able to form clusters of its own, presumably because it is still able to bind to acidic lipids such as PI(3)P, and it retains its ability to self-interact on membranes. However, essentially no co-localization with the ESCRT-II clusters was seen on the SLBs (Fig. 3, D–F), even when the concentration of the mutant was raised to 30 nM (shown in Fig. 3, D–F). Thus, the interaction occurs via the crystallographically defined ESCRT-II/VPS20 interface.

We sought to determine whether VPS20 and ESCRT-II form assemblies that interact on the molecular size scale, as opposed to large low density clusters. A single cluster FRET experiment was performed. The single cluster FRET experiment takes advantage of the fact that ~90% the Alexa488 label in ESCRT-II was located in the VPS25 subunit, and therefore within 1–2 Förster radii of the VPS20-binding site. ESCRT-II was allowed to form clusters and then rhodamine-labeled VPS20 was flowed into the chamber. Energy transfer was observed as a function of time by monitoring the intensity of fluorescence in both donor and acceptor channels (Fig. 4, A and B).

The absolute number of molecules of ESCRT-II and VPS20 was estimated from the intensity of fluorescence and the calculated labeling efficiencies. The observed average ESCRT-II: VPS20 stoichiometry of 1:2.1 ± 0.3 (data not shown) is close to the 1:2 stoichiometry suggested by the crystal structure.
the VPS20 mutant experiments and the single cluster FRET, we conclude that clustered ESCRT-II binds directly to downstream ESCRT-III member VPS20.

Clustering of ESCRT-II Is Cholesterol-dependent—The clustering of ESCRT-II binding on SLBs was assessed as a function of mol % cholesterol by TIRFM. We observed no complex clustering in SLBs containing 0–10% cholesterol (Fig. 5, A and B). At 15 mol % cholesterol, clustering was observed (Fig. 5C), but its character was more diffuse than at 25 mol % (Fig. 5D). Taken together with the observation that the EEA1 FYVE domain does not cluster on cholesterol-containing SLBs, these results are interpreted to mean that the clustering of ESCRT-II is cholesterol-dependent and depends on specific interactions among ESCRT-II complexes and specific lipid surface area. Because clustering is cholesterol-dependent, we considered whether clustering might also depend on the presence of saturated acyl chains in the lipids. We also sought to explore whether a PI(3)P with a tail composition that is thought to be more abundant in cells would support clustering. We repeated the clustering experiment with DOPI(3)P and found that the cluster formation was robust and similar to DPPI(3)P-dependent clustering (Fig. 5E).

ESCRT-II Induces Phase Separation in the SLB—Because clustering of ESCRT-II is cholesterol-dependent (Fig. 5), phase separation was assessed in the model lipid system. The phase-sensitive fluorescent dye DiD (Molecular Probes) preferentially localizes to the liquid disordered phase and was used to monitor the phase state of the lipid bilayer. First, we determined if the DiD dye was distributed evenly and was free to move laterally within the SLB. The latter was addressed using a fluorescence recovery after photobleaching experiment. A circular region of the SLB was bleached, and fluorescence was completely recovered within ~100 s. The equal distribution and quick recovery of the DiD dye demonstrate that the lipid bilayer had no phase separation prior to ESCRT assembly, consistent with the absence of clustering of the FYVE domain construct. Unlabeled ESCRT-II and VPS20-Atto488 were added to the SLB that contained DiD (Fig. 6, A–C). The ESCRT clusters that formed were found to be depleted of DiD relative to the rest of the bilayer (Fig. 5B). The lipids associated with ESCRT-II clusters are thus in a more ordered environment than their surroundings.

DISCUSSION

The energetics and geometry of vesicle budding by ESCRT-I and -II dictate that multiple copies of these complexes must assemble to drive budding. A structural model for the bud neck assembly in yeast MVB biogenesis predicted that at least six copies each of ESCRT-I and -II would be present. In human cells, MVB buds are larger than in yeast, and the corresponding assembly would thus be predicted to have at least ~12 copies. The main importance of the cluster sizes seen in this study is that they are in the range needed, as estimated from structural modeling and on energetic grounds, to drive protein clustering. Furthermore, the lipid domains observed in this study are not spontaneously formed, as demonstrated by the uniform distribution of the EEA1-FYVE construct. The lipid composition used in this study is distant on the phase diagram from the expected point of spontaneous lipid demixing (22). We attribute the clustering observed in this study to a combination of weak lateral interactions between ESCRT-II complexes, preferential interactions between ESCRT-II complexes and phosphoinositides, and the ability of the phosphoinositide/cholesterol/POPC/POPS mixture to phase-separate in response to protein binding.

ESCRT-II clusters are functionally competent to bind stoichiometrically to ubiquitin at nanomolar concentrations. The finding that monoubiquitin is competent to direct endosomal

![Image](603x792)
protein trafficking in yeast (23) was initially paradoxical given that most ubiquitin binding domains of the trafficking machinery bind to monoubiquitin with $K_D$ values of 100 μM or higher (24–26). Membrane tethering of ubiquitin, via its covalent attachment to integral membrane proteins, increases the local concentration of ubiquitin moieties on endosomal membranes. Co-clustering of 65 nm membrane-tethered ubiquitin with yeast ESCRT-0, -I, and -II was observed on GUVs, suggesting that membrane tethering provided enough of an increment in affinity to resolve the apparent paradox. The GUV experiment did not, however, address the stoichiometry of the interaction. Here, we find that at 100 nM bulk concentration, tethered ubiquitin binds with 1:1 stoichiometry to human ESCRT-II. Human ESCRT-II has only one known ubiquitin-binding site, with its GLUE domain (27, 28). These results show that human ESCRT-II contains a single ubiquitin-binding site in a functionally relevant membrane setting.

One of the fundamental and conserved functions of ESCRT-II is to couple the upstream ubiquitin-binding and membrane-budding machinery to the downstream ESCRT-III membrane scission machinery. This occurs through the binding of the two Vps25 subunits of the Y-shaped ESCRT-II complex (29, 30) to the Vps20 initiator subunit of ESCRT-III (29, 31). Both copies of the Vps25 subunit, and both Vps20 binding events, are required for function (30, 32). Yeast Vps20 undergoes an activating conformational change upon binding to Vps25 (33), which then triggers the recruitment of Snf7 and more downstream ESCRT-III subunits, leading to membrane scission (15). The interacting fragments of human VPS25 and VPS20 have been co-crystallized (19). The interacting residues shown to be conserved and essential for ESCRT function in yeast and in the in vitro GUV assays (19). Here, we have been able to demonstrate co-clustering of VPS20 with ESCRT-II on SLBs at physiologically relevant nanomolar concentrations. The interaction occurs through the structurally characterized interface, because mutations designed on the basis of the crystal structure prevent the recruitment of VPS20 to the ESCRT-II clusters. The stoichiometry of VPS20 binding to ESCRT-II was previously inferred from crystal structures and mutational impairments of function. Atomic force microscopy data were consistent with these findings but did not directly differentiate between the two interacting partners (34). Here, we have found by direct quantitation of protein levels on SLBs that VPS20 binds ESCRT-II on membranes with a 2:1 stoichiometry.

The fundamental finding in this study is that ESCRT complexes can induce lipid phase separation coupled with their self-assembly. There have been suggestions based both on mechanistic grounds and on observations in cells that motivated these experiments. The other well characterized examples of coatless budding reactions appear to involve lipid phase separation. The effect of this phase separation is to induce line tension, which at sufficiently high values can drive budding and scission on its own (35, 36). In our current view of ESCRT-mediated budding, line tension effects are not postulated to be large enough to drive budding and scission on their own. Indeed, unregulated lipid-driven budding would be undesirable for the cell and, in practice, appears to be shut down in the absence of the recruitment of ESCRTs by ubiquitinated cargo (37). We postulate that lipid phase separation is one facet of a more complex mechanism driven by the ESCRT-I, -II, and -III complexes (18).

There are physiological grounds for expecting that the ESCRTs might have a direct or indirect role in the budding of cholesterol-enriched membranes into MVBs. Impairment of various ESCRT components in cells (38, 39, 40) blocks endosomal cholesterol transport. It is particularly striking that ESCRT-0 depletion in HeLa cells appears to phenocopy depletion of the lysosomal cholesterol efflux proteins NPC1 and NPC2. Yet ESCRT-0 depletion does not affect the trafficking of these latter proteins (40), suggestive of a direct effect on cholesterol internalization in the endosome. The intraluminal vesicles of MVBs in human B lymphocytes are among the most cholesterol-rich internal membranes in these cells (41). The
Lipid Phase Separation by ESCRTs

ESCRTs contain no known cholesterol binding domains, and there have been no reports that they bind to cholesterol. It seems more likely to us that the ESCRTs directly bind to phosphoinositides, which in turn forms clusters with cholesterol, as opposed to a direct interaction. This effect is observed even with DOPlipid species, which contains unsaturated acyl chains and is thought to be more representative of cellular phosphoinositide pools.

The interplay between cholesterol and phosphoinositides in the clustering of proteins in model membranes has been explored in several other systems, and at least three different themes emerge. (i) The transmembrane domain-containing SNARE syntaxin-1A can cluster in the presence of either cholesterol (42) or an unsaturated PI(4,5)P2 (43), but the roles of these lipids in any given clustering event appear to be antagonistic (44). (ii) The dipalmitoylated polybasic protein GAP43 binds to cholesterol-rich domains via its lipid anchor and can overcome the energy barrier to bring unsaturated PI(4,5)P2 into these domains via strong electrostatic interactions with the inositol headgroup (45). (iii) The peripheral membrane protein Annexin A2, which has neither a lipid modification nor a transmembrane segment, binds electrostatically to PI(4,5)P2. Saturated (dipalmitoyl) PI(4,5)P2 couples annexin to cholesterol microdomain formation (46). The clustering phenomenon visualized here is most similar to case iii, although in this study, the clustering effect is seen even with an unsaturated phosphoinositide. It is intriguing that VPS20 is constitutively myristoylated in cells (31, 47) and that this modification is required for function yet is dispensable for high affinity membrane binding. It is tempting to speculate that a VPS20 myristoylation in cells might synergize with and strengthen the clustering and phase separation effects described here.

In conclusion, the data presented here demonstrate that ESCRTs can provoke phase separation in model membranes in the course of cluster formation. The monitoring of ESCRT clusters by TIRFM on SLBs provides a tool to directly quantify the interactions and stoichiometries of ESCRTs with each other and ubiquitin in the membrane setting. The early results for ESCRT-II cluster size and VPS20 and ubiquitin interactions are consistent with predictions from crystallographic, solution biochemical, and computational analyses. The mutual consistency of this growing body of data is encouraging with respect to current models of ESCRT mechanism in cargo sorting, membrane budding, and membrane scission.

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REFERENCES

1. Hanson, P. I., Shim, S., and Merrill, S. A. (2009) Cell biology of the ESCRT machinery. Curr. Opin. Cell Biol. 21, 568–574
2. Hurley, J. H., and Stenmark, H. (2011) Molecular mechanisms of ubiquitin-dependent membrane traffic. Annu. Rev. Biochem. 80, 119–142
3. Shields, S. B., and Piper, R. C. (2011) How ubiquitin functions with ESCRTs. Traffic 12, 1307–1317
4. McDonald, B., and Martin-Serrano, J. (2009) No strings attached. The ESCRT machinery in viral budding and cytokinesis. J. Cell Sci. 122, 2167–2177
5. Naban, J. F., Hu, R., Oh, R. S., Cohen, S. N., and Lu, Q. (2012) Formation and release of arrestin domain-containing protein 1-mediated microversicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. Proc. Natl. Acad. Sci. U.S.A. 109, 4146–4151
6. Hurley, J. H., Boura, E., Carlson, L. A., and Różycki, B. (2010) Membrane budding. Cell 143, 875–887
7. Meyers, J. R., and Audhya, A. (2012) Vesicle formation within endosomes. An ESCRT marks the spot. Commun. Integr. Biol. 5, 50–56
8. Sens, P., Johannes, L., and Bassereau, P. (2008) Biophysical approaches to protein-induced membrane deformations in trafficking. Curr. Opin. Cell Biol. 20, 476–482
9. Ewers, H., Römer, W., Smith, A. E., Bacia, K., Dmitrieff, S., Chai, W., Mancini, R., Kartenbeck, J., Chambon, V., Berland, L., Oppenheim, A., Schwarzwann, G., Feizi, T., Schwille, P., Sens, P., Helenius, A., and Johannes, L. (2010) GM1 structure determines SV40-induced membrane invagination and infection. Nat. Cell Biol. 12, 11–18
10. Trajkovic, K., Hsu, C., Chiantia, S., Rajendran, L., Wenzel, D., Wieland, F., Schwille, P., Brügger, B., and Simons, M. (2008) Ceramide triggers budding of exosome vesicles into multivesicular endosomes. Science 319, 1244–1247
11. Matsuo, H., Chevallier, J., Mayran, N., Le Blanc, I., Ferguson, C., Fauré, J., Blanc, N. S., Matile, S., Dubochet, J., Sadoval, R., Parton, R. G., Vilbois, F., and Gruenberg, J. (2004) Role of LBPA and Alix in multivesicular liposome formation and endosome organization. Science 303, 531–534
12. Wollert, T., and Hurley, J. H. (2010) Molecular mechanism of multivesicular body biogenesis by ESCRT complexes. Nature 464, 864–869
13. Hanson, P. I., Roth, R., Lin, Y., and Heuser, J. E. (2008) Plasma membrane deformation by circular arrays of ESCRT-III protein filaments. J. Cell Biol. 180, 389–402
14. Lata, S., Schoenh, G., Jain, A., Pires, R., Piehler, J., Gottlinger, H. G., and Weissenhorn, W. (2008) Helical structures of ESCRT-III are disassembled by VPS4. Science 321, 1534–1537
15. Wollert, T., Wunder, C., Lippincott-Schwartz, J., and Hurley, J. H. (2009) Membrane scission by the ESCRT-III complex. Nature 458, 172–177
16. Fabrikant, G., Lata, S., Riches, J. D., Briggs, J. A. G., Weissenhorn, W., and Kozlov, M. M. (2009) Computational model of membrane fission catalyzed by ESCRT-III. PLoS Comp. Biol. 5, e1000575
17. Boura, E., Różycki, B., Herrick, D. Z., Chung, H. S., Veger, J., Eaton, W. A., Cafiso, D. S., Hummer, G., and Hurley, J. H. (2011) Solution structure of the ESCRT-I complex by small angle x-ray scattering, EPR, and FRET spectroscopy. Proc. Natl. Acad. Sci. U.S.A. 108, 9437–9442
18. Boura, E., Różycki, B., Chung, H. S., Herrick, D. Z., Canagarajah, B., Cafiso, D. S., Hummer, G., and Hurley, J. H. (2011) Solution structure of the ESCRT-I complex by small angle x-ray scattering, EPR, and FRET spectroscopy. Proc. Natl. Acad. Sci. U.S.A. 108, 9437–9442
19. Boura, E., Różycki, B., Chung, H. S., Herrick, D. Z., Canagarajah, B., Cafiso, D. S., Hummer, G., and Hurley, J. H. (2012) Solution structure of the ESCRT-I and -II supercomplex. Implications for membrane budding and scission. Structure 20, 874–886
20. Im, Y. J., Wollert, T., Boura, E., and Hurley, J. H. (2009) Structure and function of the ESCRT-II-III interface in multivesicular body biogenesis. Dev. Cell 17, 234–243
21. Im, Y. J., and Hurley, J. H. (2008) Integrated structural model and membrane targeting mechanism of the human ESCRT-II complex. Dev. Cell 14, 902–913
22. Ivanov, V., and Mizuuchi, K. (2010) Multiple modes of interconverting dynamic pattern formation by bacterial cell division proteins. Proc. Natl. Acad. Sci. U.S.A. 107, 8071–8078
23. Veatch, S. L., and Keller, S. L. (2005) Miscibility phase diagrams of giant vesicles containing sphingomyelin. Phys. Rev. Lett. 94, 148101
24. Hicke, L. (2001) Protein regulation by monoubiquitin. Nat. Rev. Mol. Cell Biol. 2, 195–201
25. Hicke, L., Schubert, H. L., and Hill, C. P. (2005) Ubiquitin-binding domains. Nat. Rev. Mol. Cell Biol. 6, 610–621
26. Hurley, J. H., Lee, S., and Prag, G. (2006) Ubiquitin-binding domains. Biochem. J. 399, 361–372
27. Dikic, I., Wakisaka, S., and Walters, K. J. (2009) Ubiquitin-binding domains, from structures to functions. Nat. Rev. Mol. Cell Biol. 10, 659–671
28. Alam, S. L., Langelier, C., Whitby, F. G., Koira1, S., Robinson, H., Hill, C. P., and Sundquist, W. I. (2006) Structural basis for ubiquitin recognition by the human ESCRT-II EAP45 GLUE domain. Nat. Struct. Mol. Biol. 13, 1029–1030
29. Hirano, S., Suzuki, N., Slagsvold, T., Kawasaki, M., Trambiaol, D., Kato,
R., Stenmark, H., and Wakatsuki, S. (2006) Structural basis of ubiquitin recognition by mammalian Eap45 GLUE domain. Nat. Struct. Mol. Biol. 13, 1031–1032

29. Teo, H., Perisic, O., González, B., and Williams, R. L. (2004) ESCRT-II, an endosome-associated complex required for protein sorting. Crystal structure and interactions with ESCRT-III and membranes. Dev. Cell 7, 559–569

30. Hierro, A., Sun, J., Rusnak, A. S., Kim, J., Prag, G., Emr, S. D., and Hurley, J. H. (2004) Structure of the ESCRT-II endosomal trafficking complex. Nature 431, 221–225

31. Yorikawa, C., Shibata, H., Waguri, S., Hatta, K., Horii, M., Katoh, K., Kobayashi, T., Uchiyama, Y., and Maki, M. (2005) Human CHMP6, myristoylated ESCRT-III protein, interacts directly with an ESCRT-II component EAP20 and regulates endosomal cargo sorting. Biochem. J. 387, 17–26

32. Teis, D., Saksena, S., Judson, B. L., and Emr, S. D. (2010) ESCRT-II coordinates the assembly of ESCRT-III filaments for cargo sorting and multivesicular body vesicle formation. EMBO J. 29, 871–883

33. Saksena, S., Wahlman, J., Teis, D., Johnson, A. E., and Emr, S. D. (2009) Functional reconstitution of ESCRT-III assembly and disassembly. Cell 136, 97–109

34. Fyfe, I., Schuh, A. L., Edwardson, J. M., and Audhya, A. (2011) Association of the endosomal sorting complex ESCRT-II with the Vps20 subunit of ESCRT-III generates a curvature-sensitive complex capable of nucleating ESCRT-III filaments. J. Biol. Chem. 286, 34262–34270

35. Lipowsky, R. (1992) Budding of membranes induced by intramembrane domains. J. Phys. II France 2, 1825–1840

36. Baumgart, T., Hess, S. T., and Webb, W. W. (2003) Imaging coexisting fluid domains in biomembrane models coupling curvature and line tension. Nature 425, 821–824

37. MacDonald, C., Buchkovich, N. J., Stringer, D. K., Emr, S. D., and Piper, R. C. (2012) Cargo ubiquitination is essential for multivesicular body internal vesicle formation. EMBO Rep. 13, 331–338

Bishop, N., and Woodman, P. (2000) ATPase-defective mammalian VPS4 localizes to aberrant endosomes and impairs cholesterol trafficking. Mol. Biol. Cell 11, 227–239

39. Peck, J. W., Bowden, E. T., and Burbelo, P. D. (2004) Structure and function of human Vps20 and Snf7 proteins. Biochem. J. 377, 693–700

40. Du, X., Kazim, A. S., Brown, A. J., and Yang, H. (2012) An essential role of Hrs/Vps27 in endosomal cholesterol trafficking. Cell Reports 1, 29–35

41. Möbius, W., van Donselaar, E., Ohno-Iwashita, Y., Shimada, Y., Heijnen, H. F., Slot, J. W., and Geuze, H. J. (2003) Recycling compartments and the internal vesicles of multivesicular bodies harbor most of the cholesterol found in the endocytic pathway. Traffic 4, 222–231

42. Murray, D. H., and Tamm, L. K. (2009) Clustering of syntaxin-1A in model membranes is modulated by phosphatidylinositol 4,5-bisphosphate and cholesterol. Biochemistry 48, 4617–4625

43. van den Bogaart, G., Meyenberg, K., Risselada, H. J., Amin, H., Willig, K. I., Hubrich, B. E., Dier, M., Hell, S. W., Grubmüller, H., Diederichsen, U., and Jahn, R. (2011) Membrane protein sequestering by ionic protein-lipid interactions. Nature 479, 552–555

44. Murray, D. H., and Tamm, L. K. (2011) Molecular mechanism of cholesterol- and polyphosphoinositide-mediated syntaxin clustering. Biochemistry 50, 9014–9022

45. Tong, J., Nguyen, L., Vidal, A., Simon, S. A., Skene, J. H., and McIntosh, T. J. (2008) Role of GAP-43 in sequestering phosphatidylinositol 4,5-bisphosphate to Raft bilayers. Biophys. J. 94, 125–133

46. Gohkale, N. A., Abraham, A., Digman, M. A., Gratton, E., and Cho, W. (2005) Phosphoinositide specificity of and mechanism of lipid domain formation by annexin A2-p11 heterotetramer. J. Biol. Chem. 280, 42831–42840

47. Babst, M., Katzmann, D. J., Estepa-Sabal, E. J., Meerloo, T., and Emr, S. D. (2002) ESCRT-III. An endosome-associated heterooligomeric protein complex required for mvb sorting. Dev. Cell 3, 271–282