ESCRT-II coordinates the assembly of ESCRT-III filaments for cargo sorting and multivesicular body vesicle formation

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The sequential action of five distinct endosomal-sorting complex required for transport (ESCRT) complexes is required for the lysosomal downregulation of cell surface receptors through the multivesicular body (MVB) pathway. On endosomes, the assembly of ESCRT-III is a highly ordered process. We show that the length of ESCRT-III (Snf7) oligomers controls the size of MVB vesicles and addresses how ESCRT-II regulates ESCRT-III assembly. The first step of ESCRT-III assembly is mediated by Vps20, which nucleates Snf7/Vps32 oligomerization, and serves as the link to ESCRT-II. The ESCRT-II subunit Vps25 induces an essential conformational switch that converts inactive monomeric Vps20 into the active nucleator for Snf7 oligomerization. Each ESCRT-II complex contains two Vps25 molecules (arms) that generate a characteristic Y-shaped structure. Mutant ‘one-armed’ ESCRT-II complexes with a single Vps25 arm are sufficient to nucleate Snf7 oligomerization. However, these oligomers cannot execute ESCRT-III function. Both Vps25 arms provide essential geometry for the assembly of a functional ESCRT-III complex. We propose that ESCRT-II serves as a scaffold that nucleates the assembly of two Snf7 oligomers, which together are required for cargo sequestration and vesicle formation during MVB sorting.

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Introduction

The evolutionary conserved endosomal-sorting complex required for transport (ESCRT) complexes generate multivesicular bodies (MVBs), assist viral budding and promote the final membrane scission step at the end of cytokinesis. These unrelated processes all require a topologically similar

membrane fission event that is distinct from the membrane/vesicle fission associated with the formation of endocytic and secretory vesicles, which bud into the cytoplasm. ESCRT-mediated processes function in a wide variety of cellular and developmental processes and their dysfunction contributes to diseases ranging from cancer to neuro-degeneration (Raiborg and Stenmark, 2009; Saksena and Emr, 2009).

The core of the ESCRT machinery consists of a subset of the vacuolar protein-sorting gene products (VPS genes) that were first identified in yeast. A total of 17 VPS gene products assemble into five distinct sub-complexes: ESCRT-0, ESCRT-I, ESCRT-II, ESCRT-III and the Vps4 complex. These complexes are transiently recruited from the cytoplasm to endosomes. Their sequential action directs the sorting of ubiquitinated transmembrane proteins (cargo) and the inward budding of MVB vesicles into the lumen of the endosome, thereby generating MVB vesicles that are filled with cargo. Fusion of MVBs with the lysosome delivers the cargo-laden MVB vesicles into lysosomes, where they are finally degraded. ESCRT-0, I and II function early in the pathway and are recruited to endosomes as stable heteromeric complexes. Their distinct ubiquitin interacting modules bind to, collect and concentrate ubiquitinated transmembrane proteins (Hurley and Emr, 2006; Williams and Urbe, 2007). In contrast, ESCRT-III subunits are inactive monomers in the cytoplasm. ESCRT-III consists of four-core subunits: Vps20, Snf7, Vps24 and Vps2 (Babst *et al.*, 2002a). On endosomes, they assemble in a highly ordered manner to generate the transient ESCRT-III complex. Vps20 nucleates the oligomerization of Snf7. Approximately 10–20 Snf7 molecules could form a ‘ring-like’ filament that is capped by Vps24 (Teis *et al.*, 2008). The capping of Snf7 filaments by Vps24 recruits Vps2, which in turn recruits the Vps4 AAA-ATPase complex to disassemble ESCRT-III before MVB vesicle formation. Genetic and biochemical experiments suggest that ESCRT-II functions directly upstream of ESCRT-III during MVB sorting and is required for the recruitment and assembly of the ESCRT-III complex on endosomes (Babst *et al.*, 2002b). Moreover, the interaction of Vps25 with Vps20 was found to induce conformational re-arrangements in Vps20 on membranes *in vitro* (Teo *et al.*, 2004; Saksena *et al.*, 2009). As Vps20 is required to nucleate Snf7 oligomerization, the interaction of Vps25 with Vps20 is likely to be a key step that regulates the ordered assembly of ESCRT-III.

From yeast to human beings, ESCRT-II forms a trilobal complex and consists of Vps36, Vps22 and two Vps25 molecules (Babst *et al.*, 2002b; Hierro *et al.*, 2004; Teo *et al.*, 2004; Im and Hurley, 2008). Vps36 contains a GLUE domain, a ‘hub’ that mediates interaction with ESCRT-I (Vps28) (Teo *et al.*, 2006) and binds to PI(3)P (phosphatidylinositol-3-phosphate) on endosomes and to ubiquitinated cargo (Alam *et al.*, 2004, 2006; Slagsvold *et al.*, 2005; Hirano *et al.*, 2006). The GLUE domain is connected through a long flexible linker to the core

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of ESCRT-II. A tight interaction of Vps36 and Vp22 provides the scaffold for the assembly of two Vps25 molecules. One Vps25 binds to Vps36 and the other binds to Vps22 and thereby generates the characteristic Y-shape of the ESCRT-II complex. Despite our knowledge of the molecular architecture of ESCRT-II and the emerging assembly reaction of the transient ESCRT-III complex, the molecular mechanism for the ESCRT-II-dependent assembly of ESCRT-III is not understood.

Here, we show that the ESCRT-II subunit Vps25 induces a conformational change in Vps20. This conformational switch activates ('opens') Vps20, which is essential to nucleate Snf7 filaments. A mutant ESCRT-II complex with a single Vps25 molecule is sufficient for the formation of Snf7 oligomers, but not for the formation of a functional ESCRT-III complex that can sequester cargo and form MVB vesicles. Only the Y-shaped ESCRT-II complex with two Vps25 molecules (arms) provides the required geometry to induce the assembly of a functional ESCRT-III complex. It seems that during MVB sorting, ESCRT-II could simultaneously nucleate at least two Snf7 oligomers, which together execute the last steps of MVB sorting, cargo sequestration and MVB vesicle formation.

Results

ESCRT-III assembly affects MVB vesicle size

In vivo and *in vitro* experiments have shown that the ordered assembly of the ESCRT-III complex constitutes the machinery that executes the last steps of MVB sorting, cargo sequestration and MVB vesicle formation (Teis *et al*, 2008; Saksena *et al*, 2009; Wollert *et al*, 2009). It seems probable that the molecular architecture of ESCRT-III determines how MVB vesicles are formed. The first indication came from over-expression of hSnf7/hVps32 (Chmp4), which assembled into large polymeric circular arrays that deformed the plasma membrane away from the cytoplasm (Hanson *et al*, 2008). Larger ESCRT-III complexes could possibly alter the size and morphology of MVB vesicles. To directly test this idea, we modified the architecture of ESCRT-III by over-expression of Snf7/Vps32, because Snf7 seems to be key for the formation of MVB vesicles *in vivo* and *in vitro*. (Babst *et al*, 2002a; Hanson *et al*, 2008; Wollert *et al*, 2009). Over-expression of Snf7 (~20-fold, Supplementary Figure 1A) resulted in the accumulation of larger and heterogeneous Snf7 oligomers on endosomes (Supplementary Figure 1B), which caused a delay, but not a block, in MVB sorting (Supplementary Figure 1C). In wild-type (WT) cells, addition of Methionine resulted in the efficient transport of Mup1-GFP from the plasma membrane into vacuole through the MVB pathway. Interestingly, in cells that over-express Snf7, the transport of Mup1-GFP to the vacuole was delayed. Mup1-GFP first accumulated on endosomes and was then transported into the vacuole (Supplementary Figure 1C; Teis *et al*, 2008). To address how ESCRT-III with larger Snf7 oligomers would alter MVB vesicle morphology, Snf7 was over-expressed in *vma4Δ* mutant cells. These cells cannot assemble the V-ATPase complex and thus fail to acidify their vacuoles (Morano and Klionsky, 1994). As vacuolar hydrolases are less active in *vma4Δ* mutants, the contents of their vacuoles are less efficiently degraded. Unlike WT yeast cells, which have electron dense vacuoles when examined by transmission electron microscopy, the vacuoles of *vma4Δ* mutants are more transparent and MVB vesicles (15–76

MVB vesicles/vacuole/section, mean = 37 ± 15 , $n = 30$ vacuoles) can be detected in the lumen (Wurmser and Emr, 1998) (Figure 1A and B). The average vesicle diameter was 34 ± 11 nm ($n = 504$ vesicles, from 10 vacuoles) (Supplementary Figure 1D and E), which is consistent with earlier reports, suggesting an average vesicle diameter of 20–40 nm (Wurmser and Emr, 1998; Nickerson *et al*, 2006; Richter *et al*, 2007). In contrast, in *vma4Δ* mutants that over-express Snf7, fewer (2–15 vesicles/vacuole/section, mean = 7 ± 3 , $n = 30$ vacuoles) and more heterogeneous vesicles with a single limiting membrane were detected (Figure 1A and B). The average vesicle diameter was 75 ± 38 nm ($n = 315$ vesicles from 30 vacuoles) (Supplementary Figure 1D and E). To directly compare the size of MVB vesicle from *vma4Δ* mutants with *vma4Δ* mutants that over-express Snf7, the size distribution of 300 vesicles was plotted in 10 nm steps (Figure 1C). In *vma4Δ* mutants, there was a clear preference for the formation of relatively uniform vesicles with diameters ranging from 20 to 50 nm. In contrast, in the *vma4Δ* mutants that over-express Snf7, this preference was lost. The smallest vesicle had a diameter of 20 nm and the largest 360 nm. In between, the size of the vesicle diameters was evenly distributed (Figure 1C). These findings suggest that larger and more heterogeneous Snf7 oligomers on endosomes (Supplementary Figure 1B) induced the formation of fewer, but larger and more heterogeneous, MVB vesicles. It seems that Vps24-dependent capping is lagging behind Snf7 oligomerization, which results in the assembly of larger Snf7 filaments. Yet, Vps24 capping is a prerequisite for MVB vesicle formation (*vma4Δ vps24Δ*, Table in Figure 1A). In addition, loss of ESCRT-II function, which is required for the assembly of ESCRT-III (*vma4Δ vps25Δ*, Figure 1A lower panel or *vma4Δ vps36Δ*, Table in Figure 1A), blocked MVB vesicle formation. No MVB vesicles were detected in 80% of all vacuoles (24 out of 30 vacuoles). In 6 out of 30 vacuoles (20%), one abnormal vesicle was detected (Figure 1B; Table in Figure 1A). Taken together, these *in vivo* observations seem relevant for the mechanism of ESCRT-III-mediated MVB vesicle formation. During MVB sorting, ~20 Snf7 molecules, with a length of 70 Å each (Muziol *et al*, 2006) would be sufficient to delineate a circular 50 nm-sorting domain that could result in the formation of MVB vesicle with a diameter of 25 nm. It follows that longer Snf7 filaments may delineate a larger MVB-sorting domain and hence generate larger MVB vesicles. Taken together, these data strongly suggest that the function of the ESCRT-III complex is controlled by the stoichiometry of its components. In particular, the length of Snf7 filaments seems to determine the size of MVB vesicles *in vivo*. Given the uniform size of MVB vesicles, the assembly of ESCRT-III on endosomes should be tightly controlled. This raises the important question, how is the assembly of a functional ESCRT-III complex regulated?

ESCRT-II is required for Snf7 nucleation (oligomerization), but not Vps20 localization

Earlier reports suggested that ESCRT-II is required for the assembly of ESCRT-III (Babst *et al*, 2002b). To begin to understand the underlying molecular mechanism, we first tested whether ESCRT-II was essential for the recruitment of different ESCRT-III subunits to endosomes. Therefore, we used C-terminal GFP fusions (or mCherry) of ESCRT-III molecules that were generated by chromosomal integration.

Figure 1 ESCRT-III regulates the size of MVB vesicles. (A) Transmission electron microscopy of *vma4Δ*, *vma4Δ_(THD3)SNF7* and *vma4Δ vps25Δ* cells. Over-expression of Snf7 (*vma4Δ_(THD3)SNF7*) resulted in the appearance of fewer, larger and more heterogeneous luminal vesicles inside the vacuole of *vma4Δ* mutant cells. *vma4Δ vps25Δ* mutants grew very slowly. Representative cells and magnifications are shown. Size bars (500 nm and 100 nm in the magnification). The table shows the range of the number of vesicles detected per vacuole/70 nm section in different ESCRT mutants. (n = 30 vacuoles). (B) Average number of MVB vesicles/vacuole/70 nm section (n = 30 vacuoles for each sample). *vma4Δ* mutants have on average 37 ± 15 vesicles, *vma4Δ vps25Δ* have 0.3 ± 0.5 vesicles and *vma4Δ_(THD3)SNF7* have 7 ± 3 vesicles/vacuole/section. (C) Distribution of the vesicle diameter in 10 nm step (*vma4Δ*: n = 300, *vma4Δ_(THD3)SNF7*: n = 300). In *vma4Δ* mutants, most vesicle diameters range from 20 to 45 nm (black line). In *vma4Δ_(THD3)SNF7* mutants, the smallest vesicle is 20 nm and the largest vesicle is 360 nm. In between, the vesicle diameters are evenly distributed.

Figure 2 ESCRT-II is required for Snf7 nucleation. (A) Live cell microscopy of cells harbouring chromosomal integrations for *VPS20*-GFP and *SNF7*-mCherry. Size bar (5 μ m). Vps20-GFP and Snf7-mCherry co-localize on endosomes. In a *vps36Δ* mutant Vps20-GFP localizes to endosomes and Snf7-mCherry mis-localized into the cytoplasm. (B) Vps20-GFP localizes to endosomes independently of ESCRT-II (*vps36Δ*, *vps22Δ*, *vps25Δ* triple mutant). Vps20G2A-GFP did not localize to endosomes in WT cells. FM4-64 is shown in red. Size bar (5 μ m). (C) Spheroplasts of WT cells harbouring a chromosomal integration of *VPS25-Flag* or *vps36Δ* mutants were cross-linked with 5 μ m DSP. Solubilized membrane fractions (P13) were subjected to velocity sedimentation and analysed by SDS-PAGE and western blot with the indicated antibodies. *Background band.

The C-terminal GFP fusions exhibited a dominant negative phenotype and accumulated on aberrant endosomes (class E compartments), which allows easy identification of regulators of membrane recruitment (Teis *et al*, 2008). Deletion of Vps4 had no effect on the localization of Vps20-GFP or Snf7-mCherry (or Snf7-GFP) to the class E compartment (data not shown). Snf7-mCherry accumulated in large structures on endosomes. Vps20-GFP was found in the cytoplasm and on endosomes where it decorated the larger Snf7-mCherry structures (Figure 2A). Snf7-mCherry was mis-localized into the cytoplasm in *vps36Δ* (ESCRT-II) mutants (and *vps22Δ*, *vps25Δ* data not shown); yet in the same cells, the endosomal localization of Vps20-GFP was not affected (Figure 2A, second panel).

Although the ESCRT-II subunit Vps25 interacted directly with Vps20, but not with Snf7 (Teo *et al*, 2004; Saksena *et al*, 2009), ESCRT-II was essential to recruit Snf7-GFP to endosomes. Yet, the localization of Vps20 to endosomes is independent of ESCRT-II. A triple deletion of all ESCRT-II subunits (*vps22Δ*, *vps25Δ*, *vps36Δ*) did not affect the targeting of Vps20-GFP to membranes co-labelled with FM4-64 (Figure 2B). In contrast, mutation of the conserved N-terminal myristoylation site G2A (Babst *et al*, 2002a; Yorikawa *et al*, 2005) resulted in mis-localization of Vps20G2A-GFP into the cytoplasm (Figure 2B) of WT cells, suggesting that the N-terminal myristoylation of Vps20 is crucial for endosomal targeting. Thus, *in vivo* the N-terminal myristoyl-

ation and not ESCRT-II first directs Vps20 to endosomes. On endosomes, Vps20 interacts with ESCRT-II. However, without ESCRT-II, Vps20 fails to recruit Snf7 to endosomes.

In vitro, Vps25 induces conformational re-arrangement(s) in Vps20 on liposomes (Saksena *et al*, 2009). Hence, the interaction with Vps25 seems to be required for the activation of Vps20 before the nucleation of Snf7 oligomers. To directly test this idea, we determined the molecular weight of cross-linked ESCRT-III in ESCRT-II mutant cells using velocity sedimentation centrifugation. In WT cells, expressing Vps25-Flag, endosomal Snf7 cross-linked into a 450-kDa complex and did not contain detectable amounts of Vps25-F (Figure 2C). In ESCRT-II mutants (*vps36Δ* (Figure 2C), *vps22Δ* and *vps25Δ* data not shown), Snf7 did not form the 450 kDa complex, suggesting that ESCRT-II is required for the nucleation of Snf7 oligomers. Taken together, these findings strongly suggest that transient interactions with ESCRT-II (Vps25) activate Vps20, which then nucleates Snf7 oligomerization and thus initiates ESCRT-III assembly.

Vps20 activation by Vps25 (ESCRT-II) is required for ESCRT-III assembly

To better understand the molecular mechanism of ESCRT-II-dependent Vps20 activation, we next used mutational analysis of Vps20 and Vps25. Expression of the long N-terminal $\alpha 1/\alpha 2$ hairpin (aa 1–116, Vps20H1,2) of Vps20 caused a strong MVB-sorting defect (class E compartment, data not

Figure 3 Vps20 activation by Vps25 is required for ESCRT-III assembly. (A) Live cell microscopy of *vps20Δ*, SNF7-GFP cells expressing either VPS20, *vps20H1,2*, *vps20ΔH2* or *vps25ΔSNF7-GFP* cells expressing *vps25T150K*. FM4-64 is shown in red. Size bar (5 μm). (B) Spheroplasts of WT or *vps20Δ* mutants expressing the indicated Vps20 mutants were cross-linked with 5 μM DSP. Solubilized membrane fractions (P13) were subjected to velocity sedimentation and analysed by SDS-PAGE and western blot with the indicated antibodies. (C) Cartoon representation of Chmp3 hVps24 (PDB: 3FRT, (Bajorek *et al*, 2009)). Indicated are the loop region and helix α1, α2 and auto-inhibitory helix α5, and K61 of Vps20. The auto-inhibitory helix α5 was proposed to move away from the loop region, thereby releasing auto-inhibition. (D) The cytoplasm of cells expressing VPS20-Flag, VPS25-Flag or *vps20Δ*, *vps20loop-HA* or *vps20Δ*, *vps25Δ*, *vps20loop-HA* was subjected to size exclusion chromatography on an S200 column. Two fractions were pooled and analysed by SDS-PAGE and western blot with the indicated antibodies. (E) Fluorescence emission spectra of Vps20^{61-NBD} (red trace) and Vps20loop^{61-NBD} (blue trace). (F) *In vitro* oligomerization experiment. Liposomes were mixed with purified H₆-Snf7 alone, or ESCRT-II (E-II), H₆-Vps20G2A and H₆-Snf7 or ESCRT-II (E-II), H₆-Vps20loop and H₆-Snf7 (20-fold excess) and incubated for 30 min. The proteins bound to liposomes were solubilized and subjected to velocity sedimentation and analysed by SDS-PAGE and western blot with the indicated antibodies.

shown). Yet, the N-terminal α1/α2 hairpin of Vps20 was sufficient to recruit Snf7-GFP to endosomes (Figure 3A). However, the deletion of the N-terminal helix α2 (Δ55–116 aa, Vps20ΔH2) resulted in cytoplasmic mis-localization of Snf7-GFP (Figure 3A). Vps20ΔH2 is properly expressed and localized to endosomes (data not shown), but cannot nucleate Snf7 oligomerization as shown by velocity sedimentation centrifugation (Figure 3B, lower panel). Recently, the molecular details of the Vps25 and Vps20 interface have been characterized (Im *et al*, 2009). Vps25 binds specifically to helix α1 of Vps20, suggesting that this interaction could induce Vps20 activation and subsequent Snf7 oligomerization. To directly test this idea *in vivo*, we mutated critical residues in Vps25 (T150K) that were shown to be required for the interaction with Vps20 (Im *et al*, 2009). Importantly, the

Vps25T150K mutant failed to activate Vps20, which caused the mis-localization of Snf7-GFP into the cytoplasm (Figure 3A, lower panel).

Together, these findings emphasize that Vps25 binding to helix α1 of Vps20 is required for activation, whereas helix α2 of Vps20 is essential for the subsequent nucleation of Snf7 oligomers (Figure 3C). Helix α2 of different ESCRT-III proteins has been proposed to mediate the interaction between ESCRT-III molecules. Importantly, in each of the ESCRT-III proteins, helix α2 is auto-inhibited by helices α5/α6, which prevents premature assembly of ESCRT-III (Shim *et al*, 2006; Zamborlini *et al*, 2006; Bajorek *et al*, 2009; Saksena *et al*, 2009). The loop region that connects helix α1 with helix α2 has also been found to provide a crucial function. It holds the two helices α1/α2 in place and seems to position them for

proper function. Furthermore, this $\alpha 1/\alpha 2$ loop region was proposed to mediate contacts between different ESCRT-III subunits (Muziol *et al*, 2006) and/or intra-molecular contacts with the auto-inhibitory domain of $\alpha 5/\alpha 6$ (Bajorek *et al*, 2009). We reasoned that any reduction in the length of the $\alpha 1/\alpha 2$ loop would compromise the ability of the loop to hold the two N-terminal helices in place and would generate an ESCRT-III molecule with dramatic alterations in its three-dimensional folding and structure. If the intra-molecular interactions between $\alpha 1$ and $\alpha 2$ were critical for maintaining auto-inhibition, then physical displacement of $\alpha 1$ and $\alpha 2$ (by reduction in the length of the $\alpha 1/\alpha 2$ loop) would generate a more-‘active’ form of the ESCRT-III molecule and thereby result in impaired MVB-sorting function.

To directly test these hypotheses, we generated a Vps20 mutant (hereafter referred to as the Vps20loop mutant) in which we deleted residues 48–59 that lie within the $\alpha 1/\alpha 2$ loop (Figure 3C). As predicted, cells expressing the Vps20loop mutant exhibited a strong MVB-sorting defect (Figure 4B), but the localization of Snf7-GFP to endosomes was not affected (data not shown). Furthermore, the Vps20loop mutant induced the assembly of slightly larger Snf7 complexes (> 500 kDa), as shown by velocity sedimentation centrifugation (Figure 3B). This suggested that the Vps20loop mutant represented a more-active Vps20 molecule that may overcome auto-inhibition more rapidly than the WT protein. Normally, Vps20 does not interact with ESCRT-II in the cytoplasm as shown by size exclusion chromatography. Vps20 elutes at around 44 kDa, whereas Vps25 elutes together with ESCRT-II at around 150 kDa (Figure 3D) and at a second peak around 30 kDa (data not shown). Interestingly, the Vps20loop mutant eluted at a molecular weight similar to that of ESCRT-II, which was entirely dependent on Vps25 (Figure 3D). It seemed that the deletion in the $\alpha 1/\alpha 2$ loop generated a more-‘active’ form of Vps20, thereby resulting in the premature association of the Vps20 $\alpha 1/\alpha 2$ loop mutant with ESCRT-II in the cytoplasm. To directly show that the Vps20loop mutant has a different conformation in solution than the ‘auto-inhibited’ WT Vps20, we labelled recombinant Vps20 and the Vps20loop mutant with the ‘environmentally sensitive’ 7-nitrobenz-2-oxa-1,3-diazole (NBD) dye. The fact that Vps20 is a cysteine-free protein allowed us to site specifically label the WT and mutant form of Vps20 at a single site by introducing a cysteine residue, and then labelling the cysteine-containing protein with the thiol-reactive NBD. We chose to label Vps20 and the Vps20loop mutant at residue 61 because of its proximity to the $\alpha 1/\alpha 2$ loop and because we have earlier shown that residue 61 serves as a good reporter for the activation status of Vps20 (Saksena *et al*, 2009) (Figure 3C). NBD is environmentally sensitive, and its spectral properties are dramatically different in aqueous and non-aqueous environments (Johnson, 2005; Shepard *et al*, 1998). When NBD moves from an aqueous milieu (such as the surface of a soluble protein) to a hydrophobic environment (such as the non-polar interior of a membrane or protein), its emission intensity and fluorescence life time (τ) increase, whereas its wavelength of maximum emission ($\lambda_{em\ max}$) shifts to the blue (referred to as ‘blue shift’). Thus, monitoring the NBD emission intensity of two proteins labelled at the same site allows one to make conclusions regarding the environment of the NBD dye at that particular site within the two proteins (aqueous versus

Figure 4 The Vps25–Vps20 nucleation complex controls Snf7 oligomerization during MVB sorting. (A, B) Representative images of cells expressing Mup1-GFP (green). Cells were treated for 60 min with methionine and FM4-64 (red). In *snf7Δ vps20Δ* or *vps20Δ vps20PW* cells, Mup1-GFP accumulated on the E-compartment (arrowheads) and was detected at the PM (arrows). In *vps24Δ*, *vps20Δ vps20loop* and *snf7Δ snx41Δ* cells Mup1-GFP was detected only on the class E compartment. Size bar (5 μm). (C) Quantification of the fluorescence ratio (F_R) of Mup1-GFP at the PM and on the endosomes in different Vps20 mutants. High F_R indicates the accumulation of Mup1-GFP on the E-compartment; $n > 25$ cells for each mutant. (D) Canavanine sensitivity assay with WT cells and the indicated mutants. *vps20Δ* and *snf7Δ* were sensitive to 0.6 μg/ml canavanine. *vps24Δ*, *vps2Δ* and *snf7Δ snx41Δ* were more resistant to 0.6 μg/ml canavanine.

hydrophobic), and in doing so, allows one to determine whether the two proteins have different conformations around that particular site.

As shown in Figure 3E, the NBD emission intensity of Vps20^{61-NBD} (indicated by the red trace) was five-fold higher than the emission intensity of Vps20loop^{61-NBD} (blue trace) mutant labelled with NBD at the same site. Furthermore, the

lem max of the Vps20loop^{61-NBD} mutant was blue shifted compared with Vps20^{61-NBD}. This indicated that the NBD dye at residue 61 in the Vps20loop mutant is in a more aqueous environment compared with the NBD dye at residue 61 in wt Vps20 and thus supports the concept that mutations in the $\alpha 1/\alpha 2$ loop region generate a mutant Vps20 molecule that might be more active.

To address the effect of the Vps20loop mutant on Snf7 oligomerization more directly, we used *in vitro* ESCRT-III assembly reactions that we have earlier characterized (Saksena *et al*, 2009). Conditions were chosen under which purified Snf7 did not efficiently oligomerize on liposomes in the absence of its nucleator Vps20, as revealed by velocity sedimentation centrifugation of the liposome-bound proteins (Figure 3F). Upon addition of recombinant ESCRT-II and Vps20, Snf7 (present in 20-fold excess) formed higher molecular weight oligomers on liposomes. Importantly, the Vps20loop mutant induced Snf7 oligomerization more efficiently, which was readily detected because the Vps24 cap and the Vps4 complex were omitted to avoid disassembly in these assay conditions (Figure 3F).

Taken together, these findings imply that the first step in the ordered assembly of ESCRT-III is mediated by the transient binding of Vps25 to Vps20. Binding of Vps25 to Helix $\alpha 1$ of Vps20 could induce a conformational change in the loop region between $\alpha 1/\alpha 2$ that would displace the auto-inhibitory loop $\alpha 5/\alpha 6$. Consistent with this, the mutant Vps20^{PW} (P183W, P189W and P192W), which fails to displace the auto-inhibitory loop, cannot be activated and hence does not nucleate Snf7 oligomerization (Saksena *et al*, 2009). Thus, Vps25 would facilitate the release of auto-inhibition and convert Vps20 into an active nucleator, a step required to induce Snf7 oligomerization on endosomes. Our findings suggest a mechanism that helps to explain how ESCRT-II provides necessary spatial and temporal regulation for the assembly of ESCRT-III in close proximity to cargo molecules.

The Vps25–Vps20 nucleation complex controls Snf7 oligomerization during MVB sorting

To test the functional significance of the Vps25-dependent activation of Vps20, we used two different *in vivo* assays that directly measure the ESCRT-dependent vacuolar degradation of two different cell surface receptors, the methionine transporter Mup1 and the arginine transporter Can1.

First, we used quantitative live cell microscopy to examine the effect of the two different Vps20 mutants on the membrane trafficking of the methionine permease, Mup1. Mup1-GFP localized to the plasma membrane and was efficiently degraded through the MVB pathway, 60 min after addition of methionine (Teis *et al*, 2008). We have shown that loss of Snf7 oligomerization (e.g. *vps20Δ* or *snf7Δ*) (Figure 4A and B) resulted in recycling of Mup1 from aberrant endosomes back to the plasma membrane. If Snf7 oligomerization is not capped (e.g. *vps24Δ*), Mup1 is trapped on endosomes (Teis *et al*, 2008) (Figure 4A). However, in *snf7Δ vps24Δ* double mutants, Mup1-GFP was no longer trapped at the class E compartment, suggesting that the formation of Snf7 filaments is essential for cargo trapping (Supplementary Figure 2A and B). Importantly, loss of Snx41, a sorting nexin required for recycling cargo to the plasma membrane (Hettema *et al*, 2003), blocked the recycling of Mup1-GFP from the class E compartment in an *snf7Δ* mutant (Figure 4A). As the

analysis of Mup1-GFP (as well as CPS and Can1 (see Supplementary data and Supplementary Figures 2–4) traffic can distinguish different functional states of ESCRT-III assembly/disassembly, we next compared two functionally different Vps20 mutants, Vps20loop and Vps20^{PW} (P183W, P189W and P192W). Vps20loop resulted in enhanced Snf7 oligomerization (Figure 3B and F). In contrast, Vps20^{PW} restricted conformational re-arrangements of the auto-inhibitory loop in Vps20 (helices $\alpha 5/\alpha 6$) and locked the closed conformation, which did not nucleate Snf7 oligomerization (Saksena *et al*, 2009). Interestingly, in cells expressing the Vps20loop mutant, Mup1-GFP was trapped on endosomes and very little Mup1-GFP recycled back to the cell surface. In contrast, in cells expressing the Vps20^{PW} mutant, Mup1 was not trapped on endosomes and recycled back to the plasma membrane (Figure 4B). Quantification of the fluorescence intensities (I_F) on the class E compartment and at the plasma membrane allowed the calculation of a fluorescence ratio (F_R) (I_F at the class E compartment divided by the I_F at the plasma membrane). A high F_R represents predominant localization of Mup1-GFP at the class E compartment. The Vps20^{PW} mutant has a low F_R , which is comparable with *vps20Δ* or *snf7Δ* mutants (Figures 4C and 6B). In contrast, the Vps20loop mutant has a high F_R , comparable with *vps24Δ* mutants (Figures 4C and 6B). These findings showed that the Vps20^{PW} mutant, which failed to nucleate Snf7 oligomerization, permitted cargo recycling from endosomes. In contrast, the Vps20loop mutant, which hyper-activated Snf7 nucleation, trapped more cargo on endosomes.

In a complementary approach, a simple plate assay was used to determine the sensitivity of yeast to the toxic arginine analogue canavanine, which enters the cell through the arginine transporter Can1. Yeast strains impaired in endocytosis and degradation of Can1 take up excess canavanine and are, therefore, more canavanine sensitive as compared with WT cells (Lin *et al*, 2008). All ESCRT mutants are more canavanine sensitive as compared with WT cells and do not grow on 1 μg/ml canavanine (Supplementary Figure 1C). Interestingly, ESCRT mutants can be grouped into two distinct classes with respect to canavanine sensitivity. Class I mutants interfere with ESCRT-III assembly (ESCRT-II mutants as well as *vps20Δ* or *snf7Δ* mutants). All class I mutants were hyper-sensitive to canavanine and did not grow on 0.6 μg/ml canavanine (Figures 4D and 6C). Class II included mutants that block ESCRT-III disassembly (*vps24Δ*, *vps2Δ* and *vps4Δ* (data not shown)). In contrast to class I mutants, class II were less sensitive to canavanine and grew comparable with WT cells on 0.6 μg/ml canavanine (Figure 4D). Importantly, *snf7Δ vps24Δ* double mutants were hyper-sensitive class I mutants and did not grow on 0.6 μg/ml canavanine (Supplementary Figure 3C). These findings are most consistent with the idea that Snf7 oligomers trap MVB cargo on endosomes. When Snf7 oligomers cannot be formed (such as in ESCRT-II or *vps20Δ* or *snf7Δ*, *snf7Δ vps24Δ* double mutant cells), Can1 will not be degraded and recycles back to the plasma membrane. In mutants that do not cap or disassemble Snf7, the Snf7 oligomers will trap the majority of Can1 on endosomes and less Can1 will recycle back to the plasma membrane. Hence, less canavanine will enter the cells. Therefore, class II mutants are less sensitive to canavanine when compared with class I mutants. Consistently, the *snf7Δ snx41Δ* double mutant is more resistant to canavanine as

Figure 5 A single Vps25 molecule is sufficient to activate Vps20. **(A)** The cartoons depict the Y-shaped structure of ESCRT-II. ESCRT-II consists of one Vps36 (light blue), one Vps22 (dark blue) and two Vps25 molecules/arms (red). The *Vps25^{R83D}* mutation results in an 'armless' ESCRT-II complex. *Vps36^{D548R}* or *Vps22^{D214A}* mutations generate a 'one-armed' ESCRT-II complex. Live cell microscopy of Snf7-GFP in the different ESCRT-II mutants. In ESCRT-II mutants, Snf7-GFP no longer localizes to the class E compartment. Snf7-GFP eventually aggregated in cytoplasmic dots that are not FM4-64 positive. 'One armed' ESCRT-II complexes were sufficient for the recruitment of Snf7-GFP to the class E compartment. FM4-64 is shown in red. Size bar (5 μm). **(B)** Spheroplasts of cells expressing 'armless' or 'one-armed' ESCRT-II were cross-linked with 5 μM DSP. Solubilized membrane fractions (P13) were subjected to velocity sedimentation and analysed by SDS-PAGE and western blot with the indicated antibodies.

compared with *snf7Δ* mutants, because the recycling from the class E compartment to the plasma membrane was blocked. These results were complemented by quantification of the Can1-GFP fluorescence intensities on the class E compartment and the plasma membrane (Supplementary Figure 3A and B). In *vps24Δ* mutants, more Can1-GFP accumulated at the class E compartment as compared with *snf7Δ* and *snf7Δ vps24Δ* double mutants (Supplementary Figure 3A and B). Both Vps20 mutants, *Vps20loop* and *Vps20^{PW}*, caused an MVB-sorting defect (Figure 4B) and are, therefore, sensitive to 1 μg/ml canavanine (Supplementary Figure 1C). However, the *Vps20loop* mutant behaved as a class II mutant and was more resistant to lower canavanine concentration (Figure 4D). The *Vps20^{PW}* mutant was a hyper-sensitive class I mutant (Figure 4D). This finding correlated well with the proposal that the *Vps20loop* mutant was more active and favoured Snf7 oligomerization (see also Figure 3) and hence trapped more Can1 on endosomes. In contrast, the *Vps20^{PW}* mutant did not nucleate Snf7 oligomerization and more Can1 recycled back to the plasma membrane.

Taken together, these findings suggested that the ESCRT-II-mediated activation of Vps20 is a tightly regulated multi-step process that is required to control the nucleation of Snf7 oligomers.

A single Vps25 molecule is sufficient to activate Vps20

Our results strongly indicate that ESCRT-II, in particular the interaction of Vps25 with Vps20, controls the first step in the assembly of ESCRT-III. Interestingly, each ESCRT-II complex contains two Vps25 molecules (arms) in equivalent positions, which generate an unusual Y-shape (Hierro *et al*, 2004; Teo *et al*, 2004). Therefore, we next addressed how this characteristic structural feature of ESCRT-II contributes to the assembly of ESCRT-III. Well-characterized point mutations specifically disrupt the formation of the paired Vps25 arms and MVB sorting (Hierro *et al*, 2004). The *Vps25^{R83D}* mutant cannot bind to either Vps36 or Vps22, and hence resulted in the formation of an 'armless' ESCRT-II complex (Vps22, Vps36 without Vps25) (Figure 5A) (Hierro *et al*, 2004). Mutation of *Vps36^{D548R}* specifically disrupted the binding of Vps25 to Vps36, but not the binding of Vp25 to Vps22. Therefore, the *Vps36^{D548R}* mutant generated a Vps22–Vps25 'one-armed' ESCRT-II mutant complex (Figure 5A) (Hierro *et al*, 2004). Similarly, the *Vps22^{D214A}* mutant cannot bind to Vps25 and resulted in the formation of a Vps25–Vps36 'one-armed' ESCRT-II complex (Figure 5A) (Hierro *et al*, 2004). First, we examined the recruitment of Snf7-GFP to endosomes in cells expressing the 'armless' or the 'one-armed' ESCRT-II complexes. An 'armless' ESCRT-II (*Vps25^{R83D}*) no longer recruited Snf7-GFP to endosomes (class E compart-

Figure 6 Two Snf7 filaments are required to trap/encircle cargo. (A) Representative images of cells expressing Mup1-GFP (green). Cells were treated for 60 min with methionine and FM4-64 (red). In all cells, Mup1-GFP accumulates on the E-compartment (arrowheads) and is detected at the PM (arrows). Size bar (5 μm). (B) Fluorescence ratio (F_R) of Mup1-GFP at the PM and on the endosomes in different class E mutants; $n > 25$ for each mutant. (C) Canavanine sensitivity assay with WT cells and the indicated mutants. Similar to *vps20Δ* and *snf7Δ*, all ESCRT-II mutants (*vps36Δ*, *vps22Δ* and *vps25Δ*) are sensitive to 0.6 μg/ml canavanine. 'One-armed' and 'armless' ESCRT-II mutants (*Vps22^{D214A}* or *Vps25^{R83D}*) are sensitive to 0.6 μg/ml canavanine. (D) Model for ESCRT-II-mediated assembly of ESCRT-III. Both Vps25 molecules of ESCRT-II would transiently interact with two Vps20 molecules. This interaction induces conformational re-arrangements that simultaneously activate both Vps20 molecules (Step 1). Activated Vps20 then nucleates the oligomerization of two Snf7 filaments (Step 2) that are either branched (I) or run in parallel (II). Capping by Vps24 and Vps2 terminates Snf7 oligomerization (Step 3). Thereby two ESCRT-III filaments could generate an MVB-sorting domain to sequester cargo and form MVB vesicles.

ments) (Figure 5A), most likely because endosomal Vps20 cannot be activated. Surprisingly, 'one-armed' ESCRT-II complexes (*Vps36^{D548R}* or *Vps22^{D214A}*) were sufficient to activate Vps20 and recruit Snf7-GFP to endosomes (class E compartments) (Figure 5A). Next, we addressed the oligomerization status of Snf7 in cells expressing the mutant ESCRT-II complexes. Velocity sedimentation centrifugation showed that 'armless' ESCRT-II could not have triggered Snf7 oligomerization (Figure 5B). In contrast, 'one-armed' ESCRT-II complexes (*Vps36^{D548R}* or *Vps22^{D214A}*) were sufficient to induce the nucleation of a 450-kDa Snf7 filament (Figure 5B). Surprisingly, a 'one-armed' ESCRT-II complex, containing a single Vps25 molecule, is sufficient to activate Vps20. Moreover, it seems that Snf7 oligomers, which are nucleated from 'one-armed' ESCRT-II complexes, are still disassembled by Vps4, because larger Snf7 oligomers were not detected (Figure 5B). Yet, Snf7 oligomerization on endosomes is not

sufficient for MVB sorting under physiological conditions, which suggests that an intact ESCRT-II complex in some ways is required to produce functional ESCRT-III assemblies.

ESCRT-II might nucleate at least two Snf7 filaments to sort MVB cargo

It seems that ESCRT-II functions as a scaffold, in which a single Vps25 molecule is sufficient to induce nucleation of the ESCRT-III complex. However, it was clear from the earlier experiments that ESCRT-II with only one Vps25 arm is not sufficient for MVB sorting (Hierro *et al*, 2004). Therefore, we next asked how two Vps25 arms contribute to the functional assembly of ESCRT-III during MVB sorting using quantitative live cell microscopy of Mup1-GFP and the canavanine sensitivity assay (Figure 6).

All ESCRT-II mutants (*vps22Δ*, *vps25Δ* and *vps36Δ*) phenocopied *snf7Δ* and *vps20Δ* mutants; more Mup1-GFP

recycled back to the plasma membrane (Figure 6A and B). Similarly, in mutants expressing the 'armless' (Vps25^{R83D}) or 'one-armed' ESCRT-II complexes (Vps22^{D214A}, Vps36^{D548R}), Mup1-GFP was no longer sorted to the lumen of the vacuole (Figure 6A and B). In the 'one-armed' ESCRT-II mutant cells, Mup1-GFP accumulated at the class E compartment and was detected at the plasma membrane, despite the nucleation of Snf7 oligomers. Although these Snf7 oligomers are non-functional for sorting MVB cargo, they do seem to be substrates for Vps4-mediated disassembly (Figure 5B) and, therefore, cannot sequester Mup1-GFP on the endosomes. Using the Can1 plate assay showed that similar to *snf7Δ* and *vps20Δ*, all ESCRT-II mutants are class I mutants and hyper-sensitive to canavanine when compared with class II mutants (e.g. *vps24Δ*) (Figure 6C). 'Armless' ESCRT-II (Vps25^{R83D}) was a class I mutant and canavanine hyper-sensitive. Interestingly, the mutants with 'one-armed' ESCRT-II complexes (Vps22^{D14A}) (and Vps36^{D548R}, data not shown) were also phenotypic class I mutants (Figure 6C), although they supported the nucleation of Snf7 filaments. Yet, Snf7 filaments that are generated from a 'one-armed' ESCRT-II complex phenocopied loss of Snf7 filaments in the growth assay. The Vps22^{Q216A} mutant (only two amino-acid residues away from D214) was earlier shown not to affect binding of Vps25 and fully rescued the loss of Vps22 (Figure 6C). These findings indicated that ESCRT-II required both Vps25 molecules to generate a functional ESCRT-III complex. Taken together, our data indicate that both Vps25 arms in ESCRT seem to provide certain geometry during the nucleation of ESCRT-III. This might result in the assembly of at least two Snf7 oligomers (Figure 6D) that function together during MVB sorting. The first model proposes that at least two Snf7 filaments would be branched by ESCRT-II (Figure 6D, I) to define an MVB-sorting domain. Alternatively, two Snf7 filaments could be nucleated in parallel and run side-by-side on the membrane to define an MVB-sorting domain (Figure 6D, II). Maybe one Snf7 filament would sequester MVB cargo at the neck of the growing vesicle, whereas the second filament assists in membrane deformation.

Discussion

Recent findings suggested that the ESCRT-III complex bends and drives the fission of membranes in ESCRT-dependent processes, including MVB vesicle formation and the budding and release of HIV (Saksena *et al*, 2009; Wollert *et al*, 2009). The assembly of the ESCRT-III machinery on membranes is tightly regulated. In the cytoplasm, its individual subunits are kept inactive by auto-inhibition (Zamborlini *et al*, 2006; Shim *et al*, 2007). On endosomes, sequential conformational changes in the four-core ESCRT-III subunits result in the ordered assembly of the active ESCRT-III complex. Now, ESCRT-III assembly on endosomes can best be described in three distinct steps. The first step in ESCRT-III formation is the Vps25-dependent activation of the inactive Vps20 monomer on endosomes. In the second step, the activated Vps20 nucleates the homo-oligomerization of Snf7. Capping by Vps24 and Vps2 is the third step, which terminates Snf7 oligomerization (Teis *et al*, 2008; Saksena *et al*, 2009). Vps2 mediates the recruitment of the Vps4 AAA-ATPase, which then initiates the disassembly of ESCRT-III (Obita *et al*, 2007; Stuchell-Brereton *et al*, 2007). Current data indicate that Snf7

homo-oligomers form spiral-like filaments that deform membranes (Hanson *et al*, 2008). *In vitro*, individual ESCRT-III subunits assembled into filaments (Vps24), rings and sheets (Snf7) and helical tubes (hVps24, hVps2) that were disassembled by Vps4 (Ghazi-Tabatabai *et al*, 2008; Lata *et al*, 2008). Interestingly, ESCRT-III seems to generate membrane vesicles with different diameters depending on the biological context. The diameter of MVB vesicles is ~25 nm in yeast (Nickerson *et al*, 2006) and ~50 nm in HeLa cells (Doyotte *et al*, 2005). HIV-induced membrane stalks are between 50 and 100 nm in diameter and complete virions are on average 124 nm (Briggs *et al*, 2006) in diameter. The membrane tubule that connects the daughter cells just before abscission has diameter of 200 nm (Mullins and Bieseke, 1977). Thus, it seems likely that the control of ESCRT-III assembly, in particular the regulation of Snf7 oligomerization, could be one way to regulate the size of these intermediates in membrane fission. Our results indicated that the regulated assembly and disassembly of ESCRT-III complexes is required for MVB vesicle formation. ESCRT-III with an excess of Snf7 subunits is capable of generating MVB vesicles that are larger and no longer of uniform size. This finding is in good agreement with the earlier findings. Over-expressed hSnf7 assembled into large polymeric ring-like arrays that formed protruding buds and tubular evaginations at the plasma membrane of mammalian cells (Hanson *et al*, 2008). Loss of *DID2*, which has a function in coordinating Vps4-dependent ESCRT-III disassembly (Nickerson *et al*, 2006) and RNAi-mediated depletion of hVps24 resulted in the formation of slightly larger MVB vesicles (Bache *et al*, 2006). Reconstitution experiments indicated that ESCRT-III induces deformation of liposomes and can generate luminal vesicles that bud into the lumen of giant unilamellar vesicles (GUVs) (Saksena *et al*, 2009; Wollert *et al*, 2009). Interestingly, the luminal vesicles generated from GUVs had a variable diameter and were much larger than physiological MVB vesicles. Taken together, these findings emphasize a mechanistic function of ESCRT-III, in particular Snf7 homo-oligomers, during membrane deformation and vesicle scission. Therefore, it will be important to understand how the assembly of the ESCRT-III complex, in particular the nucleation of Snf7 filaments, is controlled.

ESCRT-II controls the spatial/temporal activation of ESCRT-III assembly

Genetic and biochemical experiments showed that the ESCRT-II complex is required for the formation of ESCRT-III during MVB sorting (Babst *et al*, 2002b). The ESCRT-II subunit Vps25 binds specifically to helix $\alpha 1$ of Vps20 (and not to Snf7) and induces conformational re-arrangements in Vps20 on liposomes *in vitro* (Teo *et al*, 2004; Saksena *et al*, 2009). These findings suggested that the interaction of Vps25 with Vps20 could be the first step in the assembly of ESCRT-III. Alternatively, Vps20 was found to bind to the C-terminal domain of Vps28 (Pineda-Molina *et al*, 2006). How this interaction could contribute to MVB sorting is currently not understood. Interestingly, we found that ESCRT-II is not required for the recruitment of Vps20 to endosomes. N-terminal myristoylation of Vps20 and Chmp6 (hVps20) seems to be the primary targeting signal to endosomes (Babst *et al*, 2002a; Yorikawa *et al*, 2005). However, the interaction of the ESCRT-II subunit Vps25 with helix $\alpha 1$ of Vps20 is required for the recruitment and the nucleation of

Snf7 oligomers. Although the localization of Vps20 to endosomes is not affected by loss of ESCRT-II, Vps20 fails to nucleate Snf7, suggesting that Vps25 activates Vps20. Our data suggest that Vps25 could facilitate the release of auto-inhibition by binding to helix $\alpha 1$ of Vps20 (Im *et al*, 2009) and thereby displaces the auto-inhibitory loops ($\alpha 5/6$) from $\alpha 2$, which subsequently triggers Snf7 nucleation. This idea was supported by mutations in the loop region between $\alpha 1$ and $\alpha 2$ of Vps20 (Vps20loop) and mutations in the auto-inhibitory loop ($\alpha 5$ – $\alpha 6$) (Vps20^{PW}) (Saksena *et al*, 2009). On membranes, the Vps20loop mutant more rapidly released auto-inhibition and more efficiently nucleated Snf7 oligomerization *in vitro* and *in vivo*. However, it is important to note that Vps20loop could not bypass the requirement for ESCRT-II, suggesting that the Vps20loop mutants behaved similar to a more-active Vps20 molecule. In contrast, the Vps20^{PW} mutant cannot release auto-inhibition (Saksena *et al*, 2009) and behaved similar to a loss of function mutant, which was unable to nucleate Snf7 oligomers. Hence, our data indicate a two-step activation process for Vps20. First, Vps20 is targeted to endosomes, which requires N-myristoylation. Yet, endosomal targeting is not sufficient to recruit or nucleate Snf7 polymers, but may enhance the efficiency of the interaction between Vps20 and Vps25 (ESCRT-II). Next, Vps25 induces the release of Vps20 auto-inhibition by binding to $\alpha 1$ in Vps20, which probably displaces the auto-inhibitory region ($\alpha 5/\alpha 6$). This finally results in full activation of Vps20, which then triggers Snf7 nucleation through helix $\alpha 2$ of Vps20. This activation mechanism of Vps20 seems conserved in higher eukaryotes, in which the C-terminal half of hVps25 (EAP20) bound more tightly to the N-terminal half (helix $\alpha 1/\alpha 2$) of hVps20 (Chmp6) (Langelier *et al*, 2006). As ESCRT-II binds to PI(3)P (phosphatidyl-inositol-3-phosphate) and to ubiquitinated cargo, this two-step activation mechanism of Vps20 ensures spatial and temporal control of ESCRT-III formation in close proximity to MVB cargo.

ESCRT-II could assemble at least two Snf7 filaments during MVB sorting

Similar to ESCRT-0 (Vps27/Hsc1) and ESCRT-I (Vps23, Vps37, Vps28 and Mvb12), ESCRT-II is recruited to endosomes as a preformed complex consisting of Vps36, Vps22 and two Vps25 molecules (Babst *et al*, 2002b; Hierro *et al*, 2004; Teo *et al*, 2004). One Vps25 molecule binds to Vps36 and the second Vps25 binds to Vps22. The two Vps25 arms create the characteristic Y-shape of the ESCRT-II complex. Specific mutations in either Vps22 or Vps36 disrupt the binding of one Vps25 molecule and result in the assembly of an ESCRT-II complex that contains only one Vps25 molecule. These ‘one-armed’ ESCRT-II complexes were no longer functional, which showed that both Vps25 arms are each required for MVB sorting (Hierro *et al*, 2004). Unexpectedly, we found that one-armed ESCRT-II is nonetheless sufficient to activate Vps20 and subsequently nucleate Snf7 oligomerization on endosomes. Although one Vps25 molecule is sufficient for Vps20 activation, both Vps25 molecules are required for MVB sorting. Snf7 oligomers that are nucleated from a ‘one-armed’ ESCRT-II are not functional during MVB sorting and accumulated abnormal endosomal compartments. Probably, a single Snf7 filament is not sufficient to generate a functional ESCRT-III lattice. Therefore, it is reasonable to speculate that ESCRT-II functions as a scaffold for Vps20

activation. Both Vps25 arms would provide geometry during the nucleation process that might result in the formation of two Snf7 filaments. Maybe the one filament would sequester MVB cargo at the neck of the growing vesicle, whereas the second filament assists in membrane deformation (Figure 6D). On the basis of the available ESCRT-II structures, it is difficult to speculate on the orientation of the two Snf7 filaments. Both Vps25 arms seem to be flexible (Hierro *et al*, 2004; Teo *et al*, 2004; Im and Hurley, 2008) and might undergo conformational changes when ESCRT-II binds to membranes. Interestingly, ESCRT-II as well as hVps20 (Chmp6) are dispensable for HIV budding and cytokinesis, whereas hSnf7 (Chmp4) is required for both (Martin-Serrano *et al*, 2003; Langelier *et al*, 2006; Carlton and Martin-Serrano, 2007; Morita *et al*, 2007), suggesting a different activation mechanism for ESCRT-III. During HIV budding and cytokinesis, the BRO domain of ALIX/AIP could provide an alternative activation scaffold for the formation of ESCRT-III. ALIX/AIP binds directly to hSnf7 (Chmp4) and is recruited to the site of HIV budding by p6-gag and to the mid-body by the centrosome protein 55 (Cep55) (Carlton and Martin-Serrano, 2007; Morita *et al*, 2007). This alternative, ALIX-dependent, ESCRT-III nucleation process could bypass the requirement for ESCRT-II and Vps20. As viral budding and cytokinesis do not require cargo sorting, ESCRT-III nucleated from ALIX may just be required for membrane fission. However, in the MVB pathway, ESCRT-II would provide the added function of cargo sorting and membrane deformation by regulating the nucleation of a second Snf7 filament. It seems possible that different nucleation mechanisms induce and regulate the assembly of ‘specialized’ ESCRT-III machineries during MVB sorting, cell division and HIV budding. The ESCRT-II-dependent activation of Vps20 on endosomes may be dedicated to the assembly of an ESCRT-III complex that consists of at least two Snf7 oligomers that together execute the last steps of MVB sorting, cargo sequestration and MVB vesicle formation.

Materials and methods

Materials

The mouse monoclonal antibody specific for Flag was purchased from Sigma. Rabbit polyclonal antibodies specific for Snf7 were described earlier (Babst *et al*, 1998, 2000). DSP was from Pierce and FM4-64 from Invitrogen.

Yeast strains and DNA manipulation

Saccharomyces cerevisiae strains, Media and DNA manipulations are described in Supplementary data. *vma4Δ vps25Δ* and *vma4Δ vps36Δ* mutants were grown in YNB (pH = 4.5).

In vivo cross-linking and velocity sedimentation on 10–40% glycerol gradients

These were performed as described earlier (Teis *et al*, 2008); 30 OD₆₀₀ equivalents of yeast were spheroplasted, lysed in PBS and subjected to subcellular fractionation (Babst *et al*, 1997). Membrane fractions (P13) and cytoplasm (S100) were solubilized in PBS, 0.2% Tween 20. Linear glycerol gradients (10–40%) + 0.2% Tween20 were prepared. Solubilized protein samples were loaded and spun for 210 min at 100 000 g. A total of 1 ml fractions were collected from the top and TCA precipitated.

Gel filtration

Gel filtration was performed as described earlier (Babst *et al*, 2000).

Fluorescence spectroscopy

Steady state fluorescence spectroscopy measurements were performed as described earlier (Saksena *et al*, 2009).

Microscopy and image analysis

Microscopy was performed as described earlier (Teis *et al*, 2008). The fluorescence intensity (I_F) of Mup1-GFP was measured using a 19×19 Pixel ROI on an E-compartment (I_{FE}) or at the plasma membrane (I_{FPM}) and subtracted against background (19×19 Pixel ROI outside cells). F_R was calculated $F_R = I_{FE}/I_{FPM}$. Yeast cells were labelled with FM4-64 for 10 min, washed twice and incubated for 45 min before imaging (Vida and Emr, 1995).

In vitro assembly reaction

Liposomes were generated using Folch fraction I and extruded using 100 nm filters. ESCRT-II, H₆-VPS20 and H₆-SNF7 were purified as described earlier (Saksena *et al*, 2009). Equimolar ESCRT-II and Vps20 ratios were used. Snf7 was added in 20-fold excess and incubated with liposomes for 30 min at 22°C. Liposomes were collected by 30 min centrifugation at 55 000 r.p.m. The pellet was re-suspended in solubilization buffer (PBS + 0.2% (v/v) Tween 20) and layered on top of a 10–40% linear glycerol gradient.

Transmission electron microscopy and image analysis

Cells were grown in YNB (pH = 4.5). A yeast pellet of 50 ODs was re-suspended in fix buffer (3% glutaraldehyde, 0.1 M sodium cacodylate, 5 mM calcium chloride, 5 mM magnesium chloride

and 2.5% sucrose, pH 7.4) and fixed for 1 h at room temperature. For detailed information, refer to Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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